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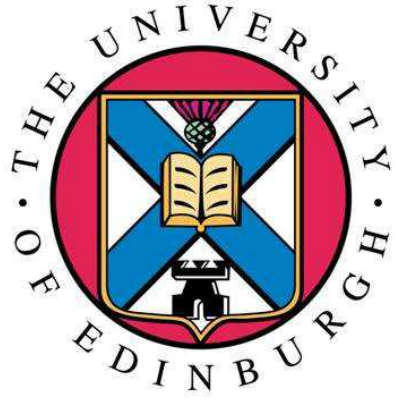

A study of carbapenem resistance in *Acinetobacter baumannii* isolates from Kuwait

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I. Dedication

I dedicate this work to my God, my family and my supervisor.

II. Abstract

Acinetobacter baumannii is a Gram-negative, non-fermenting bacillus that has developed into an important nosocomial pathogen, affecting millions of patients worldwide. The widespread ease of transmission, and ability to become multidrug resistant are some of the characteristics that, at the present time, have developed this bacterium into one of the most significant nosocomial pathogens today. The special ability it exhibits in developing resistance to a wide variety of known antimicrobial agents also helped make this a pathogen of profound importance in modern day medical microbiology. Carbapenems are used as a last resort for treating patients infected with resistant or multi-drug resistant (MDR) *Acinetobacter baumannii*. Hospitals have long served as reservoirs for the transmission of pathogenic bacteria, and this has become a problem in Kuwait. Unfortunately, very little research has been devoted exclusively to investigating *Acinetobacter baumannii* prevalence, resistance and pathogenicity in Kuwaiti Hospitals. Research on the local population in Kuwaiti Hospitals is important and beneficial to physicians, to help better diagnose and treat the infections, and prevent any outbreaks from spreading.

Aim: This study aimed to examine the resistance and identify the genotypic changes in the organism as it spreads through Mubarak Al-Kabeer Hospital.

Methods: A total of 88 *Acinetobacter baumannii* samples were collected from the Mubarak Al-Kabeer Hospital, over a three year period, 2006-2008, and they were identified phenotypically, by Vitek-2 systems, and then genotypically by PCR amplification of $bla_{\text{OXA-51-like}}$ gene. The

resistance to the carbapenems: imipenem and meropenem, was identified by use of the Minimal Inhibitory Concentration (MIC) test. Pulsed field gel electrophoresis (PFGE) was used to type the strains and classify them into clonal groups. Identification of the *bla*$_{\text{OXA-51-like}}$ gene types of each of the isolates was done via gene sequencing.

Results: All 88 isolates were identified as *Acinetobacter baumannii* by Vitek-2 system and were shown to carry a *bla*$_{\text{OXA-51-like}}$ gene. Resistance to Imipenem was found in 31.8% of the isolates, whereas resistance to meropenem was found in 23.8% of the isolates. Overall carbapenem resistance was observed in 55.7% of the total isolates, with a slight increase in resistance of isolated over the 3 years of collection. In all, there were 10 different *bla*$_{\text{OXA-51-like}}$ genes identified. The sequences of these genes suggested there was some degree of real-time evolution of the *bla*$_{\text{OXA-51-like}}$ genes during the study period. There were four main clonal clusters. There were three main European clones (*bla*$_{\text{OXA-66}}$, *bla*$_{\text{OXA-69}}$, and *bla*$_{\text{OXA-71}}$) plus a new clone with *bla*$_{\text{OXA-51-like}}$ genes with sequences clustered around the *bla*$_{\text{OXA-98}}$ gene.

Conclusion: This study has shown four major clones were found in the hospital during the study period, three of the clones were closely associated with those found in Europe and elsewhere in the world, and one new clone, containing a *bla*$_{\text{OXA-98-like}}$ gene that appears to be more prevalent in this part of Asia. The gradual increase in resistance to carbapenems over the study period warrants further attention and study of this resilient bacterium.

III. Acknowledgements

I would like to express my sincere appreciation and gratefulness to my supervisor Prof. Sebastian Amyes, for his continuous advice, supervision, valuable suggestions and guidance, during my study.

Special thanks to Kuwait University for providing me with the scholarship and support. Also to University of Edinburgh which allowed me to undertake this research.

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Finally, special thanks to my parents for their continuous encouragement.

IV. Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

V. Presentations at Conferences

1- A .R. Al-Hasan, A. Hamouda, A. A. Dashti & S. G. B. Amyes. The Emergence of the major European Clones of Carbapenem-Resistant *Acinetobacter baumannii* in Kuwait. Presented at 20th ECCMID in Vienna. (2010)

2- A .R. Al-Hasan, A. Hamouda, A. A. Dashti & S. G. B. Amyes. The Emergence of the major European Clones of Carbapenem-Resistant *Acinetobacter baumannii* in Kuwait. Presented at 8th International Symposium on the Biology of Acinetobacter in Rome. (2010)

VI. Abbreviations

Å	Angstroms
ADC	*Acinetobacter*-derived cephalosporinases
ARDRA	Amplified ribosomal DNA restriction analysis
bp	Base pairs
BSA	Bovine serum albumin
BSAC	British Society for Antimicrobial Chemotherapy
CLSI	Clinical and Laboratory Standards Institute
cm	Centimetres
DNA	Deoxyribonucleic acid
EC	European clone
ESBL	Extended-spectrum β-lactamase
G+C	Percentage of DNA consisting of guanine and cytosine bases
ICU	Intensive care unit
IMP	Imipenem
IST	Iso-Sensitest
MER	Meropenem
kb	Kilo-base pairs
L	Litre
M	Molar
Mg	Milligram
MIC	Minimum inhibitory concentration

min	Minute
mL	Millilitre
MLST	Multilocus Sequence Typing
NCBI	National Center of Biotechnology Information
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Measure of acidity or basicity of a solution
rRNA	Ribosomal-ribonucleic acid
s	Seconds
SG	Sequence group
ST	Sequence type
TBE	Tris/borate/ethylenediaminetetraacetic acid
U	Units
V	Volts
w/v	Weight by volume
µL	Microlitre
µM	Micromolar

VII. Figures and Tables

Contents

1. Introduction

The genus *Acinetobacter* has undergone major taxonomic changes over the last 30 years. *Acinetobacter baumannii* is the most significant species has and become one of the most troublesome pathogens for health care institutions the world over. Over the last 15 years, its clinical significance has been propelled by its notable ability to upregulate or acquire resistance determinants. This characteristic has made it into one of the main organisms threatening the current antibiotic era (Peleg *et al*., 2008).

Recently, reports of strains of *Acinetobacter baumannii*, which are resistant to all known antibiotics, have increased, which suggest a troubling and worrying development that should be acted on swiftly by the international health care community and local governments (Peleg *et al*., 2008). Another characteristic, alongside the increased resistance profile, which makes of *Acinetobacter baumannii* a distressing pathogen, is its ability to survive for prolonged periods of time, especially in hospital settings, thus increasing the capacity for nosocomial spread.

The organism usually targets the most susceptible and immunocompromised patients. Hospital-acquired pneumonia is considered to be the most common infection caused by this *Acinetobacter baumannii* but, more recently, infections which involve the central nervous system, skin and soft tissue, and bone have emerged as highly problematic for hospitals and health institutions (Peleg *et al*., 2008).

Scientific and Public community interest in *Acinetobacter* has been raised considerably over recent years. There have been considerable advances made in our understanding of this

interesting organism in recent years. It is important to gain a brief understanding of the relevant clinical, microbiological, as well as the epidemiological characteristics of *Acinetobacter baumannii*, which is the most the most clinically relevant and wide spread species (Peleg *et al*., 2008; Brisou and Prevot., 1954).

1.1. History of the Genus *Acinetobacter*

The history of the genus *Acinetobacter* dates back to the 1911, when Dutch microbiologist Beijerinck, described an organism named *Micrococcus calcoaceticus* that was isolated from soil by enrichment in a calcium acetate-containing minimal medium (Brisou and Prevot., 1954).

The current genus designation, *Acinetobacter* (from the Greek ακινητο [akinetos], which means: non-motile), was proposed by Brisou and Prevot in 1954 to separate the motile from the non-motile microorganisms within the genus *Achromobacter*.

In 1968, this genus designation became more widely accepted. Baumann *et al*. first published a complete survey and concluded that the different species listed belonged to a single genus, this was when the name the name *Acinetobacter* was first proposed. Further sub-classification into different species based on phenotypic characteristics was not possible (Baumann *et al*., 1968). All these discoveries resulted in the official acknowledgment of the genus *Acinetobacter* by the Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria in 1971 (Peleg *et al.*, 2008).

The genus *Acinetobacter* genus was first listed in the 1974 edition of *Bergey's Manual of Systematic Bacteriology* (Peleg *et al.,* 2008), with the description of a single species, *Acinetobacter calcoaceticus (*ATCC 23055).

1.2.Taxonomy

The genus *Acinetobacter*, as is currently defined, is a Gram-negative, strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase-positive, oxidase-negative bacteria with a DNA G/C content of between 39% and 47%. Based on more recent taxonomic data, it was proposed that members of the genus *Acinetobacter* should be classified in the new family *Moraxellaceae* within the order *Gammaproteobacteria*, which includes the genera *Moraxella*, *Acinetobacter*, *Psychrobacter*, and related organisms (Peleg *et al*., 2008, Rossau *et al*., 1991).

A major breakthrough, however, in the long and complex history of the genus was achieved in 1986 by Bouvet and Grimont, who based on DNA-DNA hybridization studies, distinguished 12 DNA (hybridization) groups or genospecies, some of which were given formal species names, including *A. baumannii*, *A. calcoaceticus*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, and *A. lwoffii* (Peleg *et al*., 2008, Bouvet and Grimont, 1986). More recently, 10 additional *Acinetobacter* species were described, including 3 species of human origin, *A. parvus*, *A. schindleri*, and *A. ursingii* (Nemec *et al*., 2001; Nemec *et al*., 2003) and 7 species isolated from activated sludge (recovered from sewage plants), namely, *A. baylyi*, *A. bouvetii*, *A. grimontii*, *A. tjernbergiae*, *A. towneri*, *A. tandoii*, and *A. gerneri*, increasing the actual number of validly described (genomic) species to 31.(Peleg *et al*., 2008, Carr *et al*., 2003).

1.3.Species Identification

Acinetobacters can be identified presumptively to the genus level as: Gram-negative, catalase-positive, oxidase-negative, non-motile, non-fermenting, coccobacilli. They are short, plump, Gram-negative rods that are difficult to destain and may be misidentified as either Gram-negative or Gram-positive cocci.

Acinetobacter species of human origin grow well on solid media that are regularly used in clinical microbiology laboratories and hospitals, such as sheep blood agar or Tryptic Soy Agar, at 37°C. These organisms form smooth, sometimes mucoid, grey/white colonies.

A. calcoaceticus-A. baumannii complex colonies resemble those of *Enterobacteriaceae*, with a diameter of 1.5 to 3 mm after overnight culture, whereas most of the other *Acinetobacter* species produce smaller and more translucent colonies. Unlike the *Enterobacteriaceae*, some *Acinetobacter* species outside the *A. calcoaceticus-A. baumannii* complex may not grow on McConkey agar (Peleg *et al.*, 2008).

Many methods have contributed to the better understanding of the clinical and epidemiological significance of *Acinetobacter* species during recent years, unfortunately, they are too laborious to be applied in daily diagnostic microbiology, and are used mainly in reference laboratories (Peleg *et al.*, 2008).

More recent developments include the identification of *A.baumannii* by detection of the *bla*$_{\text{OXA-51-like}}$ carbapenemase gene intrinsic to this species (Turton *et al*., 2006), and a simple PCR-based method described by Higgins *et al*. that exploits differences in their respective *gyrB* genes to rapidly differentiate between *A. baumannii* and *Acinetobacter* genomic species 13TU (Higgins *et al.,* 2007).

Species identification with manual and semi-automated commercial identification systems that are currently used in diagnostic microbiology, such as the API 20NE, VITEK-2, Phoenix, and MicroScan WalkAway systems, remain problematic (Bernards *et al.,* 1995, Bernards *et al.,* 1996, Hawley *et al.,* 2007, Peleg *et al.,* 2008).

This can be explained in two main parts: the limited database content, and because the substrates used for bacterial species identification have not been customized specifically to identify acinetobacters. *Acinetobacter baumannii*, *Acinetobacter* genomic species 3, and *Acinetobacter* genomic species 13TU are uniformly identified as *A. baumannii* by the most widely used identification systems (Peleg *et al*., 2008).

The need for species identification of acinetobacters in routine clinical laboratories has been questioned by some researchers (Gerner-Smidt *et al.,* 1991); however, from a clinical and infection control point of view, it is important to distinguish between the *Acinetobacter baumannii* group and Acinetobacters outside the *A. baumannii* group since the latter organisms rarely cause infection complications. Also, these organisms are usually sensitive to a wide range of antimicrobials, and infections caused by these organisms are usually benign.

However, from a research standpoint, clinical studies using appropriate and accurate methods for species identification of acinetobacters, including those within the *A. baumannii* group, are necessary to increase our knowledge of the pathogenicity, epidemiology, and clinical implications of the species of this diverse genus.

1.4.Natural Habitats

Members of the genus *Acinetobacter* are considered ubiquitous organisms; acinetobacters can be recovered, after enrichment culture, from virtually all samples obtained from soil or surface water (Baumann, 1968). Similar studies have been carried to show that *Acinetobacter baumannii* is also ubiquitous in nature (Fournier and Richet, 2006).

An epidemiological survey was performed to investigate the colonization of human skin and mucous membranes with *Acinetobacter* species, and showed that up to 43% of non-hospitalized individuals were colonized with these organisms (Seifert *et al*., 1997). An interesting study done in Hong Kong, by Chu *et al*. who found 53% of medical students and new nurses to be colonized with acinetobacters in summer versus 32% in winter (Chu *et al*., 1999). The seasonal variability in skin colonization could be attributed to the seasonal variation seen in clinical samples of *Acinetobacter baumannii* (McDonald *et al.,* 1999).

As these two studies show, the chances of growth and survival of *Acinetobacter baumannii* in warm environments, such as Kuwait, are higher, and could therefore help explain the increased reports of *Acinetobacter baumannii* in warm climate regions such as the middle-east.

1.5.Antimicrobials Agents and Acinetobacter

There is great concern regarding the rise in the number of infections caused by *Acinetobacter baumannii,* especially since over the recent years many difficulties are being faced in administrating effective antimicrobial treatment. The inherent properties of *Acinetobacter baumannii,* such as chromosomally encoded β-lactamases, as well as an effective permeability barrier (Peleg *et al.,* 2008)., coupled with the ability to acquire and maintain resistance determinants on their mobile genetic elements, have rigorously decreased the number of effective antibiotics that can be used against the bacterial infections, and in some cases it was completely ineffective (Valencia *et al*., 2009).

Due to these characteristics, there has been a gradual elimination of the effectiveness of the aminoglycosides, cephalosporins, penicillins, tetracyclines, and quinolones as treatment options for many infections of *Acinetobacter baumannii*. Consequently, we are left with carbapenems as the only sustainable group of antibiotics that may currently be successfully used to treat infections of *Acinetobacter baumannii*; mainly due to their low toxicity and good activity. Carbapenems are a subgroup of the β-lactam antibiotics and have the broadest spectrum of activity within this group (Peleg *et al.,* 2008).

Imipenem was the first carbapenem to be approved for use, in 1985; which was a stable derivative of thienamycin, the antibiotic discovered in the 1970s and produced by *Streptomyces cattleya*. About twenty years later it was followed by meropenem – 1993 and more latterly by ertapenem - 2001, and doripenem - 2007 (Livermore, 2009).

1.6.Classes of Antimicrobial Agents

β-lactams: Each class of antibiotics differs from the others in their mode of action. The mode of action of β-lactam antibiotics is by inhibiting cell wall development in the bacteria. This is done by de-activating enzymes, which are located in the bacterial cell membrane during the 3rd stage of manufacturing the peptidoglycan in cell wall synthesis. This acts as the main stress-bearing layer of the bacterial cell wall. Once the peptidoglycan is no longer created, the bacterial cell wall is usually destroyed, which ultimately leads an inability to maintain the osmotic pressure leading to the destruction of the bacterial cell as a whole.

These β-lactams do not inhibit a single enzyme, but a family of related enzymes. These penicillin binding proteins, four to eight in different bacteria, are all involved in different steps in the final stages of cell wall synthesis (Spratt and Cromie, 1988).

Protein synthesis inhibitors: Macrolides, chloramphenicol, clindamycin, aminoglycosides, tetracyclines and oxazolidinones are other class of antibiotics. These mainly act by inhibiting bacterial growth by interfering with protein synthesis (Pestka, 1971). By and large, protein

synthesis inhibitors have little effect on infections caused by *Acinetobacter* spp, largely by their inability to penetrate the cell wall.

DNA gyrase inhibition is another form of bacterial inhibition, found mainly by quinolones. Once the antibiotic blocks DNA synthesis and prevents the supercoiling of the DNA, the bacterial cell ceases to reproduce (Smith, 1986). Although used initially against *A. baumannii*, there efficacy has reduced they are no longer used (Peleg *et al.*, 2008).

RNA and folate inhibitors: Action of antibiotics in the rifampicin group act to inhibit DNA-directed RNA polymerase. Once this is done, the bacterial cell can no longer create proteins and is ultimately inhibited (Wehrli *et al*., 1968).

Some synergistic combinations have also been used, most notably the combination of trimethoprim and sulphonamides, which both act on the synthesis of tetrahydrofolic acid. The value of this combination over trimethoprim alone is yet to be proven (Coates *et al*., 2002). The difficulty that these compounds have in penetrating *A. baumannii* has ensured that they have not been used extensively against it.

Carbapenems: Carbapenems are also β-lactam antibiotics. They were originally developed from thienamycin, a naturally-derived product of *Streptomyces cattleya* (Birnbaum *et al.*, 1985). Imipenem and meropenem have the broadest spectrum of activity, they can act on both aerobic and anaerobic bacteria, with a high success rate, and therefore these β-lactams have become the

antibiotics of choice for the treatment of many bacterial infections, especially multidrug resistant *Acinetobacter baumannii* (MDR-AB) (Livermore and Woodford, 2006).

There are 4 main differences between penicillins and carbapenems:

1. Carbapenems have a carbon atom instead of sulphur in the five-membered ring.
2. This ring is unsaturated in the carbapenems.
3. The orientation of the hydrogen atoms on the rings differ in the two structures.
4. The main side chain in the penicillin is at position 6 whereas it may be on the membered ring in the carbapenems (Figure.1)

These differences mean that the shape of carbapenem significantly differs from that of penicillin, thus making it much more stable to β-lactamases.

The structures of carbapenems are similar to that of penicillins, the main difference being that the sulphur atom in position 1 of the structure is been replaced with a carbon atom, hence the name, carba-penems (Kumagai *et al*., 2002).

Carbapenems are able to retain activity against Gram-negative bacteria, even when they have become resistant to other β-lactam antibiotics such as the cephalosporins. In Gram-negative bacteria this has largely resulted from the emergence of the extended spectrum β-lactamases (ESBLs) or those which hyperproduce the AmpC-type cephalosporinase (Livermore, 1995). Neither group of enzymes is capable of hydrolyzing the carbapenems.

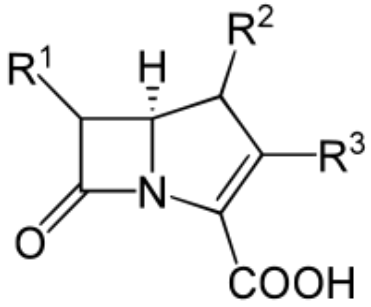


Figure 1. Showing the general structure of Carbapenems

1.7.Carbapenem resistance in *Acinetobacter baumannii*

There has been a worldwide recognition of the increase in carbapenem resistance among *Acinetobacter baumannii*. This represents an important event, particularly as the more resistant the bacteria evolve into, the harder it will be to treat and cure patients infected with them (Coelho *et al*., 2004).

This class of antibiotics includes: imipenem, meropenem, doripenem, ertapenem, and razupenem. They are parenteral synthetic β-lactams that are derived from thienamycin, an antibiotic produced by *Streptomyces cattleya* (Livermore, 2009).

There are two intrinsic enzymes, AmpC-type and OXA-51, which are considered as the most important types of β-lactamases, in almost all *Acinetobacter baumannii* isolates. The expression of the Amp-C type cephalosporinase at the basal level does not decrease the sufficiency of the expanded spectrum cephalosporins (Bou *et al.*, 2000).

Additionally, insertion sequence IS*Aba1* which are present upstream of *bla* Amp-C gene enhances the expression of this β-lactamase in *Acinetobacter baumannii* (Heritier *et al*., 2006).

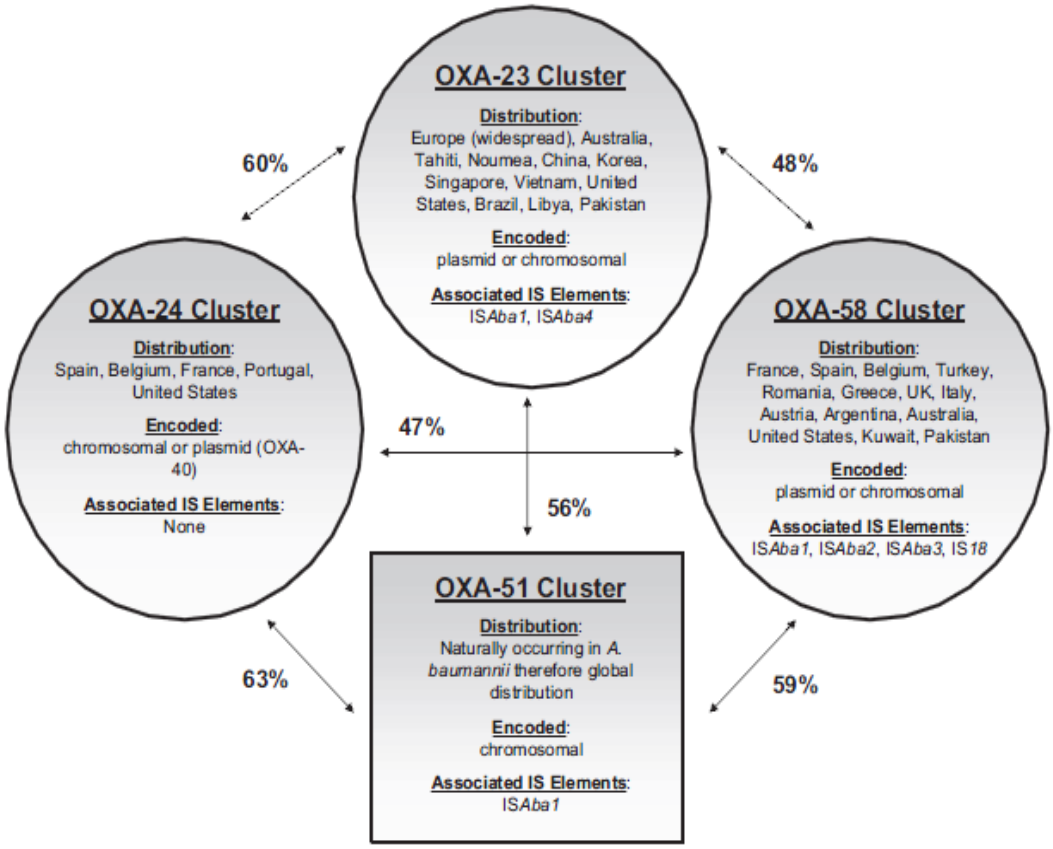


Figure 2. A Summary of the distribution and genetic context of the OXA-type enzymes in *Acinetobacter baumannii*. Each arrow and corresponding percentages correspond to the degrees of amino acid homology between the enzyme clusters. The enzyme clusters within large circles signify the acquired enzyme types, in contrast to the naturally occurring OXA-51 cluster within the large square. (taken from Peleg *et al.*, 2008)

A summary of OXA-type enzymes in *A. baumannii* is shown in Figure 2.

The first identified OXA-type enzyme with carbapenem-hydrolyzing activity was from a clinical *A. baumannii* strain isolated in 1985 from Edinburgh, Scotland (Paton *et al*., 1993).

1.8.Acquisition of oxacillinases (class D enzymes)

To date, over 227 different variants of class D OXA-type β-lactamases have been investigated on the protein level (Genebank, 2011).

The oxacillinase groups are unique β-lactamases and mainly occur in Gram-negative bacteria. Initially, they were thought to be plasmid-mediated only but since the identification of the OXA-51 β-lactamase gene, located on the chromosome of an *A. baumannii* strain from Argentina (Brown *et al*., 2005), the source of these plasmid enzymes has now been identified.

Oxacillinases commonly hydrolyze oxacillin faster than classical penicillins such as benzyl penicillin, yet, the efficiency of hydrolytic class D β-lactamases (CHDLs) against carbapenems is very low, up to 1000-fold compared with MBLs (Poirel and Nordmann, 2006b).

The first report of imipenem resistant *Acinetobacter baumannii* was reported in 1993 (Urban *et al*., 1993). At around the same time, the discovery of the first novel group of narrow spectrum OXA-type β-lactamases was made. The enzyme, which was called OXA-23, was found to have carbapenem hydrolysing activity, although the strain was obtained before the use of imipenem in 1985 in the same hospital (Paton *et al*., 1993; Donald *et al*., 2000).

1.9.OXA-types (β-lactamase)

The OXA-type carbapenem hydrolysing class D β-lactamases in *Acinetobacter baumannii* are classified into four subgroups: OXA-23-like, OXA-40-like, OXA-51-like and OXA-58-like (Brown and Amyes, 2006). Also note that, the third sub group of OXA-51-like is intrinsic and naturally located in all *Acinetobacter baumannii* (Heritier *et al*., 2005).

1.9.1. OXA-23 Group

The first family is OXA-23, formerly named ARI-1, and was identified in 1995 in Scotland and afterwards renamed to OXA-23, due to its genetic characterization. The OXA-23 group also includes OXA-27 and OXA-49. The first identification of OXA-27 was made in Singapore, while OXA-49 was in China; both of them were identified from single carbapenem resistant *Acinetobacter baumannii* strains rather than outbreaks (Turton *et al*., 2005). OXA-23 was identified a few years later, in *Acinetobacter baumannii* isolates in Brazil (Dalla-Costa *et al*., 2003), China (Yu *et al*., 2004) and Singapore (Coelho *et al*., 2004). See (Figure 3)

1.9.2. OXA-40 Group

The second family is the OXA-40, which was identified in Spain, from a highly carbapenem-resistant *Acinetobacter baumannii* strain sharing 63% & 60% amino acid identity with OXA-51/69 and OXA-23 respectively (Bou *et al*., 2000). This group also includes the variants OXA-24, OXA-25, OXA-26, and OXA-72. These variants of OXA-25, OXA-26 and OXA-40 were

identified in strains from Portugal, Spain, and Belgium afterwards. (Afzal-Shah *et al*., 2001), while the OXA-72 *Acinetobacter baumannii* strains were identified in Thailand (Walther-Rasmussen and Hoiby, 2006). (See Figure. 3)

1.9.3. OXA-51 Group

The third family was first identified by Brown *et al*. (2005) from a strain isolated in Argentina. This enzyme, which was called OXA-51, was characterized from two clones of *Acinetobacter baumannii*. OXA-51 shared a 63% amino acid identity with subgroups 1 and 2. Other members of the OXA-51 enzyme group were soon found around the world; they were found in carbapenem resistant strains and each enzyme shared 98% to 99% identity with OXA-51 (Brown and Amyes, 2006).

The identifications of these variants were based on the OXA-51-like enzyme and the construction of this group revealed three clusters of OXA-66, OXA-69 and OXA-98 of closely related enzymes. (See Figure. 3)

1.9.4. OXA-58 Group

The fourth family has only one member, OXA-58, where the first identification of OXA-58 was made in France in 2003. It has also been isolated from strains in Argentina, Kuwait and southern England (Coelho *et al*., 2004). OXA-58 has the ability to hydrolyze penicillins and Imipenem significantly and, furthermore, OXA-58 is also active against the majority of cephalosporins

(Poirel *et al*., 2005). OXA-58 has a low percentage of amino acid identity compared with the other oxacillinases, (48 and 47%) amino acid identity with OXA-23 and OXA-40, respectively, 59% with OXA-51and <50% with other CHDLs (Brown and Amyes, 2006). See Figure 3

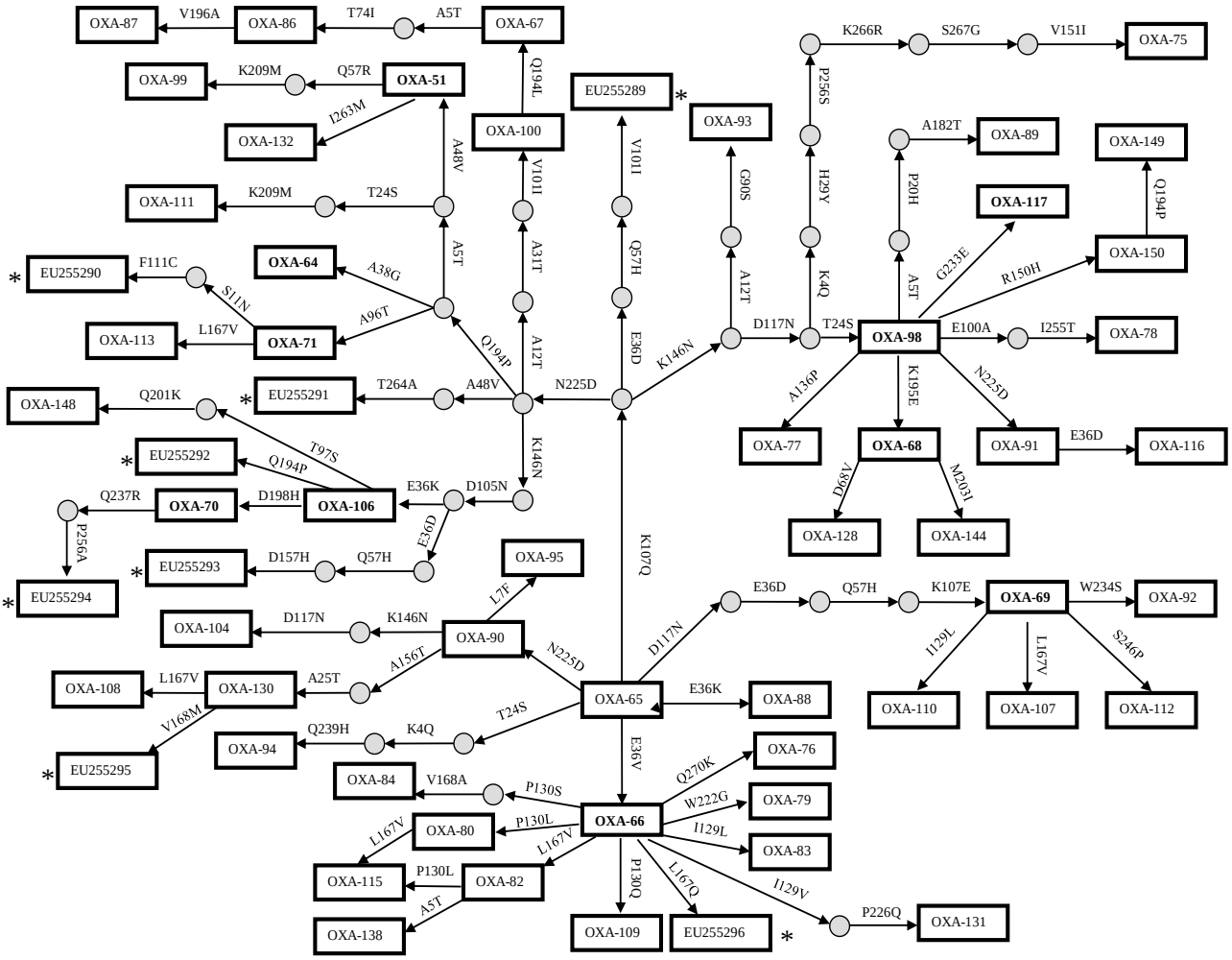


Figure 3. Modified OXA-51-like linkage map showing all related OXAs. (modified from Evans *et al*., 2007)

1.10. Clinical Laboratory Detection of Carbapenemases

A variety of β-lactamases produced by *Acinetobacter baumannii* are capable of hydrolyzing carbapenems (Queenan and Bush, 2007).

Phenotypic tests for evaluating the presence of serine carbapenemases (OXA-type) in *Acinetobacter baumannii* have not yet been described. The most frequently used methods for detecting MBLs have been disk approximation methods comprising meropenem (Figure. 4) and imipenem (Figure. 5) plus EDTA (Gales *et al*., 2003).

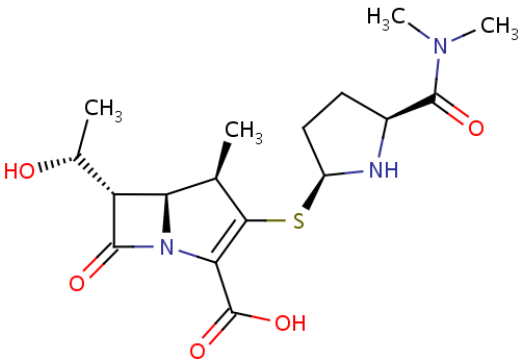


Figure. 4 Meropenem

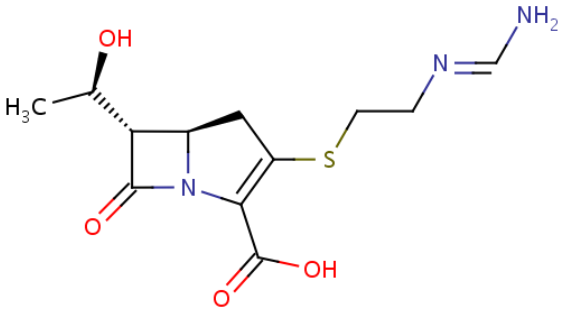


Figure. 5 Imipenem

1.11. Natural Habitat of *Acinetobacter*

The majority of *Acinetobacter* species generally can be found and isolated from environmental samples, mainly in the soil, or from non-clinical human samples such as skin. There are many members of the genus which are usually considered to be environmental organisms (Peleg *et al*., 2008). A study of water and soil samples from California, 28 of 30 soil samples and 29 of 30 water samples were culture-positive for *Acinetobacter* spp. (Baumann, 1968). The microbial communities from the extreme environments of six Andean lakes were studied, five different *Acinetobacter* isolates were identified, one of which was determined to be *Acinetobacter johnsonii* (Ordonez *et al*., 2009).

Acinetobacter calcoaceticus isolates have been discovered in the soil surrounding plant roots. Their symbiotic relationship was shown to, which promoted plant growth *in vitro* (Peix *et al*., 2009). Another species: *Acinetobacter baylyi*, which was also isolated from the soil, was shown to be able to acquire plant DNA material through horizontal gene transfer (Pontiroli *et al*., 2009). An isolate of *Acinetobacter baumannii*, recovered from oil-contaminated desert soil in Kuwait, and was shown to be an oil degrading bacterium (Obuekwe *et al*., 2009).

In a series of studies that examined the association of *Acinetobacter* spp. with humans, a wide range of results were found. The study of skin carriage from 192 healthy humans in London, UK, an *Acinetobacter* carriage rate of 44% was discovered, *Acinetobacter* genospecies 15BJ accounted for 12.5%, with *Acinetobacter lwoffii* accounted for 61% of the isolates and *Acinetobacter radioresistens* accounted for 8% (Berlau *et al*., 1999). Similar research was done

in Cologne, Germany, where it was found that 40 healthy people had an *Acinetobacter* spp carriage rate of 42.5%, with 58% of isolates accounted for by *Acinetobacter lwoffii*, 20% by *Acinetobacter johnsonii*, and 10% by *Acinetobacter junii* (Seifert *et al*., 1997). Similarly, a study of skin carriage by medical students and nurse students in Hong Kong found the *Acinetobacter* carriage rate to be 37.8%, but *Acinetobacter* genospecies 3 was the most widespread accounting for 32% of isolates, while *Acinetobacter* genospecies 13TU the second most common, accounting for around 12% (Chu *et al*., 1999). It is probable that the difference in the skin flora observed in the study from Hong Kong may be due to the study group being in contact with hospitalized patients, where carriage of *Acinetobacter* species 3 and 13TU is more widespread. While it is apparent that the environment of many of the members of the genus *Acinetobacter* is either aquatic or soil, there is dispute as to the natural habitat of a few of its species: *Acinetobacter baumannii*, *Acinetobacter* genospecies 13TU, and also *Acinetobacter* genospecies 3 - which are not usually found in environmental samples, but predominantly in hospital environments and clinical samples.

1.12. *Acinetobacter baumannii* in the Middle East

There have been numerous outbreaks of multidrug-resistant *Acinetobacter baumannii* reported in Middle Eastern hospitals, namely in Kuwait, Iraq, Iran, Israel, Egypt, Bahrain, Qatar, Saudi Arabia, and the United Arab Emirates. All have documented a variety of carbapenemases, with varying degrees of resistance (Peleg *et al*., 2008, Jamal *et al*., 2009, Alsutlan *et al*., 2009).

The map in figure 6 below shows the countries in the Middle East that have reported outbreaks of carbapenem-resistant *Acinetobacter baumannii* (Peleg *et al*., 2008).

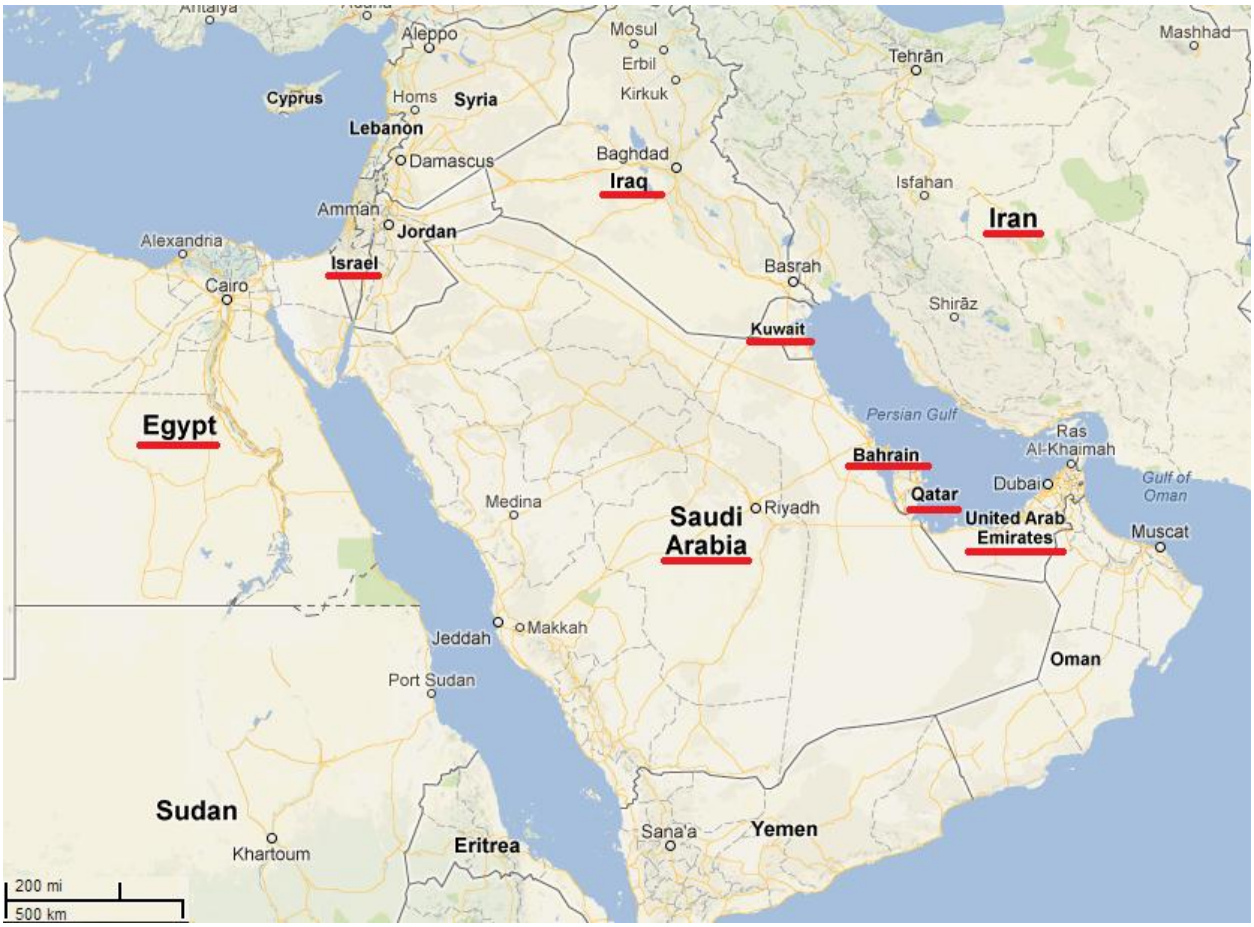


Figure 6. Countries in the Middle East underlined in red are ones that have reported outbreaks of carbapenem-resistant *Acinetobacter baumannii*. (Image courtesy of Google Earth, 2012)

1.13. Community-Acquired Acinetobacter Infections

Some of the members of the *Acinetobacter* genus have emerged to prominence relatively recently, mainly due to the increasing number of human infections for which they are responsible. Many members of the *Acinetobacter* genus have been identified as the cause of infections, nevertheless, there are three species that are implicated in causing infections far more frequently. These species are *Acinetobacter baumannii*, *Acinetobacter* genospecies 3 (more recently called *Acinetobacter pittii*) and *Acinetobacter* genospecies 13TU more recently called *Acinetobacter nosocomialis*).

The infections from *Acinetobacter baumannii* are of a particular problem, since they can be very difficult to treat. In general, *Acinetobacter* species are opportunistic pathogens, and usually only cause infections in people of whom are immunocompromised, or suffer from other immune debilitating diseases from a previous underlying condition (Falagas *et al*., 2007).

Reports of infections by *Acinetobacter* species in the community have been reported, even though very infrequently. Upon reviewing these reports, it was discovered that in the great majority of community cases, patients were diagnosed with pneumonia; while the remaining cases were diagnosed as bacteraemia infections.

The majority of patients were found to have severe conditions such as: chronic obstructive pulmonary disease, diabetes mellitus, renal disease. Smokers and excessive alcohol consumers were also amongst those patients. The infections were just about all reported to be caused by

Acinetobacter baumannii; however due to the difficulty in identifying the species within the genus, particularly between members of the *Acinetobacter baumannii -Acinetobacter calcoaceticus* complex, it may be possible that other species were also involved.

The mortality associated with infections was very high, at 56%, though this is possible to be an over-estimation, since many mild cases of *Acinetobacter* infections would not normally be reported. Also, the bulk of the reports originated from areas with climates that are tropical and subtropical. Whether these resulting conditions in the reports were due to climatic or social factors, it's still not clear (Falagas *et al*., 2007).

1.14. Hospital-Acquired Acinetobacter Infections

Whilst community-acquired *Acinetobacter* infections are infrequent, hospital-acquired infections are far more common, and of a far bigger concern. Infections are associated with immunocompromised patients, with infection rates regularly being in their highest in intensive care units (ICUs) and in surgical wards. The organism that typically causes pneumonia associated with the mechanical ventilators, and also bloodstream infections, following invasive procedures (Peleg *et al*., 2006).

Acinetobacter baumannii, *Acinetobacter pittii* and *Acinetobacter nosocomialis* are the three species which are mostly frequently implicated in cases of hospital-acquired infection. Epidemiological surveys have typically identified *Acinetobacter baumannii* to be, by far, the

most frequent causative organism of infections, although there reports of higher rates of isolation of *Acinetobacter pittii* have been made (Boo *et al*., 2009, Lim *et al*., 2007, Chen *et al*., 2007).

Acinetobacter pittii and *nosocomialis* have a propensity of sporadically causing infections. Fortunately, they are relatively susceptible to antimicrobials. On the other hand, the same cannot be said of *Acinetobacter baumannii*. The mortality rates for *Acinetobacter* species other than *Acinetobacter baumannii* have been found to be very low, between 7% and 18% respectively (Choi *et al*., 2006, Seifert *et al*., 1994b).

Conversely, the mortality rates of patients infected with *Acinetobacter baumannii* has been reported as being between 8% and 43% (Falagas *et al*., 2006). Furthermore, the duration of stay in the hospital in association with *Acinetbacter baumannii* infections was considerably longer than that for other *Acinetobacter* species (Choi *et al*., 2006).

Acinetobacter baumannii outbreaks are often clonal, and the spread of clones or lineages can be seen both nationally and internationally. A great number of global infections can be attributed to three of these epidemic lineages, that have been termed European clones I, II and III (Van Dessel *et al*., 2004, Dijkshoorn *et al*., 2007).

There is an continuing infection control problem in the UK, which is related to the circulation of several clonal lineages of *Acinetobacter baumannii*, termed the South-East clone (SE clone), OXA-23 clone I and OXA-23 clone II (Coelho *et al*., 2006).

It is still unclear what the reasons are that make some lineages of *Acinetobacter baumannii* particularly successful, and allows them to spread internationally; certainly part of the answer is likely to be because of the rather high level of antimicrobial resistance shown in this bacteria, and also by its ability to acquire and maintain resistance determinants. It is for the reasons previously mentioned that of all of the species belonging to the *Acinetobacter* genus; it is *Acinetobacter baumannii* that is the organism that gives us the greatest cause for concern.

1.14.1. Acinetobacter Pneumonia

In most cases, the majority of *A. baumannii* isolates are from the respiratory tracts of hospitalized patients. In most circumstances it is difficult to distinguish between the upper airway colonization and true pneumonia. There is no doubt, however, that true ventilator-associated pneumonia (VAP) due to *A. baumannii* occurs (Peleg *et al*., 2008).

Community-acquired pneumonia due to *A. baumannii* has been described from tropical regions of Asia (Peleg *et al*., 2008). Most typically, the disease occurs during the rainy season among people with a history of alcohol abuse, and some cases may sometimes require admission to an ICU (Anstey *et al*., 2002).

1.14.2. Bloodstream Infections

In a large study of nosocomial bloodstream infection in the United States (1995–2002), *A. baumannii* was the 10th most common aetiologic agent, being responsible for 1.3% of all monomicrobial nosocomial bloodstream infections (0.6 bloodstream infection per 10,000

admissions) (Wisplinghoff *et al*., 2004). *A. baumannii* was a more common cause of ICU-acquired bloodstream infection than of non-ICU-ward infection.

One hundred and two patients had bloodstream infections at sites treating U.S. military members injured in Iraq or Afghanistan from 1 January 2002 and 31 August 2004 (CDC, 2004).

1.15. Traumatic Battlefield and Other Wounds

Acinetobacter baumannii is frequently isolated from wounds of combat casualties from Iraq or Afghanistan (Yun *et al*., 2006). These reports could prove valuable as they suggest the possibility of a link between desert locations in the Middle East, and the prevalence of *Acinetobacter baumannii* infections, since dust storms are a natural phenomenon there, as well as top soil layers that are displaced and dispersed via tanks and helicopters, that could easily reach flesh wounds and increase the chances of infection to wounded individuals.

1.16. Treatment of *Acinetobacter baumannii* infections

The treatments available for bacteria that are innately resistant to many classes of antibiotics are decreasing. The dependability of effective treatment for *Acinetobacter* infections has become very restricted (Pieroni, 1997). On the other hand, carbapenems such as: imipenem and meropenem are considered to be the drug of choice to treat the infections caused by this bacterium.

Non-rational use of carbapenems used in treating patients has caused an increase in the overall resistance of *Acinetobacter baumannii* to this group of antibiotics (Livermore and Woodford, 2000). Nevertheless, if used prudently, then their efficacy could be ensured for a longer time (Gales *et al*., 2001).

Colistin is another option; which seems to be relatively secure and effective for the treatment of patients with infections caused by multi-drug resistant *Acinetobacter baumannii* (Kroeger *et al*., 2007, Pantopoulou *et al*., 2007). Tigecycline has also been tested and successfully used as treatment against *Acinetobacter baumannii* infections (Al-Sweih *et al*., 2009). Occasionally, aminoglycosides could also be used in combination with an effective β-lactamase with fluoroquinolone or rifampin (Bergogne-Berezin and Towner, 1996). In general, carbapenems, colistin, minocycline and tigecycline remain the best and only solutions against *Acinetobacter* infections.

Prevention, as the saying goes, is the best medicine. This type of precautionary approach would readily help decrease the chances of infection in hospital wards. Washing hands is one of the most important methods for preventing infection, where 30% of nosocomial infection could be avoided (Bourn, 2000).

1.17. Typing methods for *Acinetobacter baumannii*

Typing of *Acinetobacter* is of great importance for distinguishing the sources and modes of spreading for these bacteria. Typing is even of greater importance during an outbreak, because it is used to determine if the organism responsible is a clone or an unrelated strain, as well as revealing the source of the outbreak (Towner *et al*., 1991).

In 1987, the earliest attempts to type *Acinetobacter* spp were performed. They were based on biotypes and antibiotypes (Bouvet and Grimont, 1987). Since then, several biotyping systems have been applied to type *Acinetobacter* spp. One of the systems relied upon biochemical profiles; with all the results scored as either positive or negative, and then were used to compare strain types.

Another bio-typing technique consisted of five active tests for dividing *A. baumannii* isolates into 19 biotypes. This method was used to type the related genomic species 3 and 13TU (Bouvet *et al*., 1990). However these often prove unreliable.

1.18. Pulsed-Field Gel Electrophoresis

Even in the face of sequence-based methods that are now readily available and are challenging PFGE as the gold standard for typing of many bacterial species, for *Acinetobacter*, PFGE still remains the reference method of choice. It is a relatively laborious method, which requires several days before generating a typing result, but the

required equipment is now standard not only in most reference laboratories but also in hospital- based microbiology laboratories. Basically, Pulsed Field Gel Electrophoresis (PFGE) is restriction analysis, which is generated from intact chromosomal DNA, to compare the fingerprints of different strains following digestion with endonucleases; such as the *Apa*1 enzyme (Seifert *et al*., 1994a). Usually, the restriction endonuclease *Apa*I is used to restrict the intact chromosomal DNA of the *Acinetobacter baumannii* isolates (Bou *et al*., 2000).

PFGE is costly, and requires a lot of equipment, taking several days to prepare the DNA and subsequently digests it with the endonuclease. The process is easy to handle, readable, replicable, and provides highly discriminatory results which give useful epidemiological information (Gouby *et al*.,1992).The resulting chromosomal fragments are separated by electrophoresis, and fingerprint profiles are compared visually or using specialized computer programs that also allow the storage of profiles in a database.

As with other comparative typing systems that are based mainly on a side-by-side comparison of molecular fingerprint patterns of a limited number of strains, comparisons between different laboratories have been a problem with PFGE, but recent studies show that with adequate standardization of protocols, inter-laboratory reproducibility can be achieved (Seifert *et al*., 2005).

This approach would permit the recognition of epidemic strains and the early detection of multi-hospital or nationwide outbreaks, particularly if cases are geographically separated. Nowadays, there are other methods, such as MLST, that are found to be optimal for large scale typing (Seifert *et al*., 2005).

1.19. Aims of the thesis

The main objectives of this study were to:

- Compare the validity of phenotypic identification of *Acinetobacter baumannii*.
- Study the infiltration of carbapenem resistance into *A. baumannii* in Kuwait.
- To investigate the clonal relationship between multi-resistant *A. baumannii* strains isolated in Kuwait.
- Identify why particular clones are prevalent.

2. Materials and Methods

2.1. Strain collection

A total of 120 clinical bacterial isolates were collected from the Mubarak Al-Kabeer Hospital in Kuwait - during a period of 2 years - from 2006 to 2008. Several outbreaks occurred during those years and samples were collected accordingly. The sources were mainly from blood, urine, pus swab, endotracheal, rectal swab, and skin. The non-repeated samples were collected from a variety of patients of differences in age, sex, and nationality. All isolates collected were cultured and stored in both long term and short term methods (see below). Once all the samples were collected, they were shipped to the UK and stored at the University of Edinburgh, where further research and tests were carried.

2.2.Short Term Storage

Short term storage method is mainly used for working with the isolates on a weekly basis, and in this form it was possible to ship them to the UK with minimal loss to the viability of the bacterial isolates. The isolates were sub-cultured on MacConkey agar (Oxoid, Basingstoke) and then incubated at 37 °C for a period of 24 hours. The next day, a single colony was taken using a sterile loop and inoculated into IST broth, which was put in an orbital shaker and allowed to grow over night at 37°C. With the help of a pipette, 900µL of the overnight culture was added to 100µL sterile glycerol in sterile plastic tubes and were stored at -20°C for short-term use and storage.

2.3.Long Term Storage

The method used for storing the isolates, long-term, was the Cryobank System Beads (Kalyx Biosciences Inc, Orlando, USA). This was done by taking a single freshly grown colony, which was grown overnight at 37°C, on MacConkey agar and added to the Cryobank System vial. The vials contained a collection of beads that secured the bacterial strains for storage. This is a very successful for long-term storage of bacterial isolates. In accordance to the manufacturer's instructions, all the strain containing vials were stored in a -80 °C freezer. When required the bacteria were removed by taking out a single bead without disturbing the rest.

2.4.Media and Chemicals

All the antibiotic agents used were purchased from the Sigma-Aldrich Company Ltd (Poole, UK) unless otherwise stated. All media were purchased, in powdered form, from Oxoid (Basingstoke, UK). The broths and agar plates were prepared according to the manufacturer's instructions. They were sterilized at 121°C/15 psi for 15 minutes by autoclaving. Normal saline was prepared by dissolving 0.85 g of NaCl in 100mL of distilled water (D/W) and sterilized by autoclaving.

2.4.1. 10X TAE buffer preparation:

The preparation of 10X TAE buffer was comprised of: Trizma base (Sigma) (48.44g) of and 3.72g of EDTA (Ethylene diamine tetra acetic acid) disodium salt dehydrate 99% were dissolved in D/W with the addition of 11.4 ml glacial acetic acid to give a final volume of one litre. The pH

was adjusted to 8.0 with 1M HCl and the solution was sterilized. For gel running, a dilution of 1:10 was made to a final concentration of 1X and used for gel electrophoresis.

2.4.2. 10X TBE buffer preparation:

The preparation of 10X TBE Buffer was comprised of: Tris-base (108g) and 55g of boric acid were dissolved with 40mL of 20mM EDTA in one litre of D/W. The pH was adjusted to 8.0 with 1M HCl and the solution was sterilized. 0.5X TBE buffer was also used for the running of PFGE gels.

The isolates collected were subjected to two types of identification methods: Phenotypic and Genotypic.

2.5.Phenotypic Identification

2.5.1 VITEK-2 System

All the isolates were screened and identified via the VITEK-2 System (BioMerieux, Marcy L'Etoile, France) in Kuwait, in accordance to the manufacturer's instructions. This is a phenotypic type of identification which depends on biochemical reactions to identify the isolates.

One hundred and twenty strains were inoculated onto MacConkey agar (Oxoid) plates and then incubated overnight at 37°C. A single isolated colony was used for identification by the phenotypic VITEK-2 Systems method, done according to the manufacturer's instructions (BioMerieux, Marcy L'Etoile, France).

2.6.Genotypic Identification

2.6.1. Polymerase chain reaction (PCR)

PCR reagents:

All PCR reagents were performed using Promega reagents (Promega, Southampton, UK), GoTaq Flexi DNA Polymerase (5u/µl) in 5x green or colourless buffer, $MgCl_2$ solution (25mM) and deoxynucleoside triphosphates (dNTP) / PCR nucleotide mix (10mM each). Sterilized distilled water was used as the dilutents.

All PCR reactions unless otherwise indicated were performed in 50µl of 1.5mM (2µl) $MgCl_2$, 0.1mM (1µl) dNTPs, 1X reaction buffer (10µl), 0.05mM (0.5µl) each primer, 1µl template and 0.2 units of *Taq* in sterilized distilled water.

All the PCRs that were performed were done in a total volume of 50µL, with reagents supplied by Promega (Promega, Southampton, UK) according to the manufacturer's guidelines.

The primers that were used in this study were supplied by Eurofins MWG operon, UK. They were diluted to a concentration of 100pmol/µL as stock standards. 12.5pmol of primer concentration was used for each PCR reaction. PCR amplification of *bla*$OXA_{51\text{-like}}$ gene was carried out on all the samples, in accordance to the conditions set by Brown *et al* (2005).

2.6.2. Extraction of DNA

Following overnight sub-culture on MacConkey agar at 37^oC, DNA extraction was performed by boiling 5 identical colonies in 50 µL of sterile distilled water for about 15-20 minutes in eppendorf tubes. The eppendorf tubes were then centrifuged and the supernatant was used as the DNA template.

PCR Primer data:

The primers used for the PCR reaction were to amplify the *bla*$_{\text{OXA-51-like}}$ gene. The primers OXA-69 Forward and OXA-69 Reverse were used for the PCR. The primers and expected fragment size were:

OXA-69F	F 5'-CTAATAATTGATCTACTCAAG-'3
OXA-69R	R 5'-CCAGTGGATGGATGGATAGATTATC-'3

The expected size was 975 base pairs.

2.6.3. Gel electrophoresis and staining

Agarose gels (1.5%) (Gensieve LE agarose, Flowgen, UK) were prepared in 1X TAE buffer and run at 100V for the preparations of plugs. 0.7% gels were prepared for the running of plasmid DNA and they were run at 60V for 19hrs for better resolution. All the gels were stained in a 100 mL of gel red (Biotium, US) staining solution (50 µL gel red dissolved in 100ml of 0.1M NaCl) and visualized by the Gel Doc system.

2.7.Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was performed on all isolates identified genotypically as *Acinetobacter baumannii* isolated from the Mubarak Al-Kabeer Hospital in Kuwait, in accordance to the procedure described by Seifert *et al*. (2005). The isolates were inoculated onto Iso-Sensitest (IST) agar

(Oxoid, Basingstoke) and incubated overnight at 37°C in ambient air. A loopful of bacteria was removed from the agar surface with a sterile plastic loop and suspended in a glass or polystyrene round bottomed tube containing 2.5 ml of cell suspension buffer (100 mM Tris, 100 mM EDTA, pH 8.0). Each cell suspension was adjusted to give a cell density of approximately 10^9 cells/ml. This was performed by using the spectrophotometer. The pellet was resuspended by vortexing, and the cell suspension was incubated at 55°C for 10 min a water bath. An aliquot of 25 µL proteinase K (20-mg/ml stock solution in ultrapure water) was added, and the suspension was mixed gently by inverting the tube two to four times. An equal volume of melted 1% CHEF genomic agarose (Bio-Rad, UK)-1% sodium dodecyl sulphate (Fisher Scientific, UK) in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was added to the cell suspension, and the suspension was mixed gently by inverting the tube 10 to 12 times. The agarose-cell suspension mixture was immediately dispensed into the wells of reusable plug moulds. The agarose plugs were allowed to solidify at room temperature for 5 min and at 4°C for another 5 min. A single plug was then transferred to disposable screw-cap 50-ml polypropylene tubes containing 5 ml of cell lysis buffer (50 mM Tris, 50 mM EDTA [pH 8.0], 1% sarcosine) and 25 µL of proteinase K (20 mg/ml stock solution). Lysis was performed at 55°C in a water bath for two hours with. After lysis, the buffer was carefully removed and the plugs were washed five times (15 min/wash) at 55°C (two times with sterile ultrapure water and three times with TE buffer; 10 ml for each washing step) in a water bath. The water and TE buffer were preheated at 50 to 55°C before each washing step. After the last wash, the TE buffer was poured off and 10 ml of fresh TE buffer was added to each tube. If the plugs were not used on the same day then they were refrigerated. For the restriction of the genomic DNA a single slice of the plug (4.0 by 5.5 mm) was cut with a scalpel or razor blade and transferred to a microcentrifuge tube containing 200µL of the

restriction buffer with 30U *Apa*I (Promega, Southampton, UK). The plug slices were incubated at 37°C for 24 hours in a water bath. 1.0% gels were prepared in 0.5X TBE buffer for PFGE and the gels were run with the plugs in CHEF apparatus with an initial pulse 5 sec and final pulse 25 sec, 200V for 20 hours at 14°C . The gel was stained with gel red on the completion of run. Cluster analysis was performed by the unweighted pair group method with mathematical averaging (UPGMA), and DNA relatedness was calculated by using the band-based Dice coefficient with a tolerance setting of 1.5% band tolerance and 1.5% optimization setting for the whole profile. Gel analysis was performed using the BioNumerics v4.0 software (Applied Maths, Sint-Martins-Latem, Belgium). A value of 75-80% was chosen as the threshold for the establishment of clonal relatedness of the unknown isolates. The banding patterns were also interpreted according to the criteria suggested by Tenover *et al*. (1995).

2.8.Minimum Inhibitory Concentration Test

The isolates were tested for their susceptibility to the two major carbapenems: imipenem (IPM) (Merck, Sharp & Dohme Ltd, Hertfordshire, UK) and meropenem (MEM) (AstraZeneca, Cheshire, UK).

After overnight culturing on MacConkey agar, a single colony was suspended using a sterile loop in 5 mL of Iso-sensitest broth (Oxoid, Basingstoke) and grown overnight at 37°C/180 rpm in an orbital shaker. The minimum inhibitory concentrations (MICs) were determined by the agar double dilution technique in Iso-sensitest agar in accordance specification of the British Society for Antimicrobial Chemotherapy (BSAC) methodology (British Society for Antimicrobial Chemotherapy, Version 10.2, 2011). The results were interpreted according to the BSAC

guidelines. *Pseudomonas aeruginosa* NCTC 10662, *Escherichia coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571 were used as control strains.

2.9.Sequencing of PCR products

The amplification of the PCR products were purified using a PCR cleanup kit (Qiagen, West Sussex, UK) and an ABI3730 capillary sequencer (Applied Biosystems, Warrington, UK). OXA-69 products were used for the complete sequencing map of the *Acinetobacter baumannii* samples. The DNA sequences were then analyzed using the Chromas, Multalin, EXPASY and BLAST software. Eventually, the sequences were compared to the published sequences of *A. baumannii* using an online search facility (http://www.ncbi.nih.gov/BLAST). The amino acid sequences were translated using the EXPASY translate website (http:www.expasy.ch/tools/dna.html).

3. Results

The first stage of the experiment was to validate the phenotypes of the bacterial isolates, both phenotypically and genotypically. The first phase was to identify them phenotypically using the VITEK-2 system. Only the positive isolates were then subjected to a genotypic type of identification, using PCR (Polymerase Chain Reaction) of OXA-69 primer that exhibits bands at 975bp, which are used to verify and solidify the results. Once the identification was completed, the samples were sequenced. The resulting sequences were then run through BLAST (Basic Local Alignment Search Tool) on the PubMed website http://blast.ncbi.nlm.nih.gov/.

All the sequence results were then recorded and tabulated in the results below. Typing of the isolates was performed to identify the clonality and relatedness in all of them, this was done by the PFGE (Pulsed-Field Gel Electrophoresis) method, and the resulting dendogram was enlisted in the results below.

3.1. Phenotypic Identification

3.1.1. VITEK-2 System

The Vitek-2 System (BioMerieux, Marcy L'Etoile, France) is a phenotypic system of identification, which depends on the biochemical reactions between the bacterial isolates suspended in their respective tubes of solution, and the media in the VITEK-2 Identification Cards, to identify the isolates.

In the first stage of the experiment, 120 sample isolates were inoculated onto MacConky agar (Oxoid) plates and then incubated overnight at 37°C. A single colony was then taken and suspended into solution. The turbidity of the bacterial suspension was adjusted with VITEK Densichek (bioMerieux) to match the McFarland 0.5 standard in 0.45% sodium chloride. Then the VITEK 2 ID-GN (Gram Negative) card and the bacterial suspension tubes were manually loaded into the VITEK-2 system. Following steps on the software were done according to the manufacturer's instructions (BioMerieux, Marcy L'Etoile, France).

Of the 120 samples, 110 were identified as *Acinetobacter baumannii* positive by the VITEK-2 system. The results are recorded below on Table 1.

The Date, Sex, and Source of each positive sample were also recorded on Table 1.

Table 1. The VITEK-2 system reported the results for *Acinetobacter baumannii* detection as follows:

Sample No.	Date	Sex	Source	*A. baumannii* Detection
1	2006	M	Rectal Swab	+ve
2	2006	M	ICU	+ve
3	2006	M	Rectal Swab	+ve
4	2006	F	Endotracheal	+ve
5	2006	M	Urine	+ve
6	2006	M	Tip	+ve
7	2006	M	Urine	+ve
8	2006	F	Urine	+ve
9	2006	F	Blood	+ve
10	2006	M	Blood	+ve
11	2006	M	Blood	+ve
12	2006	M	Tip	+ve

13	2006	M	Blood	+ve
14	2006	M	Blood	+ve
15	2006	M	Blood	+ve
16	2006	M	Urine	+ve
17	2006	F	ICU	+ve
18	2006	F	Blood	+ve
19	2006	M	Blood	+ve
20	2006	M	Endotracheal	+ve
21	2006	F	Blood	+ve
22	2006	M	Blood	+ve
23	2006	F	Tip	+ve
24	2006	F	Tip	+ve
25	2006	M	Pus Swab	+ve
26	2006	M	Tissue	+ve
27	2007	M	Tissue	+ve
28	2007	F	Urine	+ve
29	2007	F	Rectal Swab	+ve
30	2007	F	Tip	+ve
31	2007	M	Blood	+ve
32	2007	M	Blood	+ve
33	2007	M	Blood	+ve
34	2007	M	Blood	+ve
35	2007	M	Skin Swab	+ve
36	2007	M	Blood	+ve
37	2007	M	Blood	+ve
38	2007	F	Blood	+ve
39	2007	F	Endotracheal	+ve
40	2007	M	Endotracheal	+ve
41	2007	F	Endotracheal	+ve
42	2007	F	Endotracheal	+ve
43	2007	M	Urine	+ve
44	2007	F	Pus Swab	+ve
45	2007	M	Endotracheal	+ve
46	2007	F	Endotracheal	+ve
47	2007	M	Sputum	+ve
48	2007	M	Blood	+ve
49	2007	M	Blood	+ve
50	2007	M	Aspiration Fluid	+ve
51	2007	M	Rectal Swab	+ve
52	2007	M	Pus Swab	+ve
53	2007	F	Pus Swab	+ve
54	2007	F	Pus Swab	+ve
55	2008	M	Blood	+ve
56	2008	M	Endotracheal	+ve
57	2008	M	Blood	+ve

58	2008	F	Blood	+ve
59	2008	F	Blood	+ve
60	2008	M	Urine	+ve
61	2008	M	Blood	+ve
62	2008	F	Blood	+ve
63	2008	M	Tip	+ve
64	2008	M	Blood	+ve
65	2008	F	Endotracheal	+ve
66	2008	F	Pertonial	+ve
67	2008	F	Rectal Swab	+ve
68	2008	M	Blood	+ve
69	2008	M	Urine	+ve
70	2008	F	Pus Swab	+ve
71	2008	F	Blood	+ve
72	2008	M	Endotracheal	+ve
73	2008	M	Tissue	+ve
74	2008	M	Pus Swab	+ve
75	2008	M	Blood	+ve
76	2008	F	Tip	+ve
77	2008	M	Tip	+ve
78	2008	M	Endotracheal	+ve
79	2008	F	Urine	+ve
80	2008	F	Blood	+ve
81	2008	F	Endotracheal	+ve
82	2008	F	Blood	+ve
83	2008	F	Blood	+ve
84	2008	F	Blood	+ve
85	2008	F	Endotracheal	+ve
86	2008	F	Tip	+ve
87	2008	F	Aspiration Fluid	+ve
88	2008	F	Blood	+ve
89	2008	M	Stool	+ve
90	2008	F	Urine	+ve
91	2008	F	Blood	+ve
92	2008	F	Stool	+ve
93	2008	F	Urine	+ve
94	2008	F	Urine	+ve
95	2008	M	Blood	+ve
96	2008	F	Stool	+ve
97	2008	F	Urine	+ve
98	2008	F	Blood	+ve
99	2008	F	Blood	+ve
100	2008	M	Urine	+ve
101	2008	F	Urine	+ve
102	2008	F	Blood	+ve

103	2008	F	Sputum	+ve
104	2008	M	Blood	+ve
105	2008	M	Urine	+ve
106	2008	F	Blood	+ve
107	2008	F	Sputum	+ve
108	2008	F	Tip	+ve
109	2008	M	Sputum	+ve
110	2008	F	Blood	+ve

According to Table 1. The statistics that can be derived from the samples, which were analyzed via VITEK-2, are as follows:

Percentage of Female patients with positive *Acinetobacter baumannii* = 49.1%

Percentage of Male patients with positive *Acinetobacter baumannii* = 50.9%

The number of positive samples for each year was also calculated and presented in a bar chart below in figure 7. The data is as follows:

Total number of positive samples for the year 2006 = 26 (18 males, 8 females)

Total number of positive samples for the year 2007 = 28 (17 males, 11 females)

Total number of positive samples for the year 2008 = 56 (21 males, 35 females)

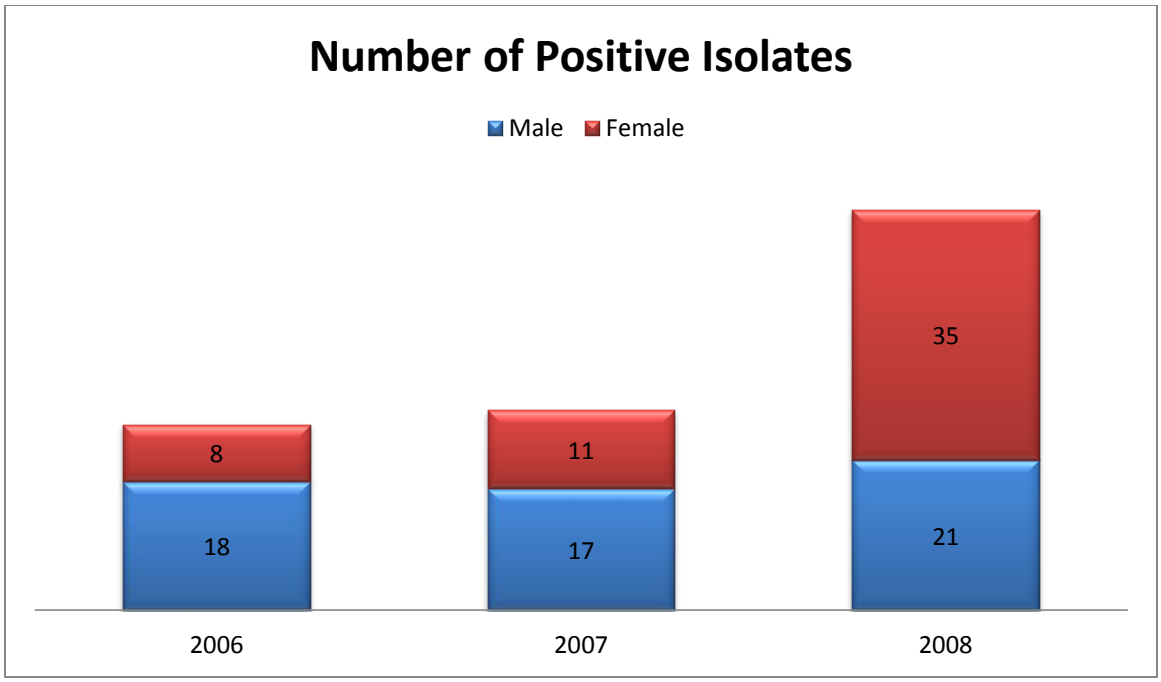


Figure 7. Bar Chart Showing the number of positive samples isolated from Mubarak Al-Kabeer Hospital during the years 2006, 2007, 2008.

According to figure 7 we could clearly observe a significant increase in the number of females that tested positive for *Acinetobacter baumannii* infection according to the VITEK-2 system in the year 2008, in comparison to the previous years.

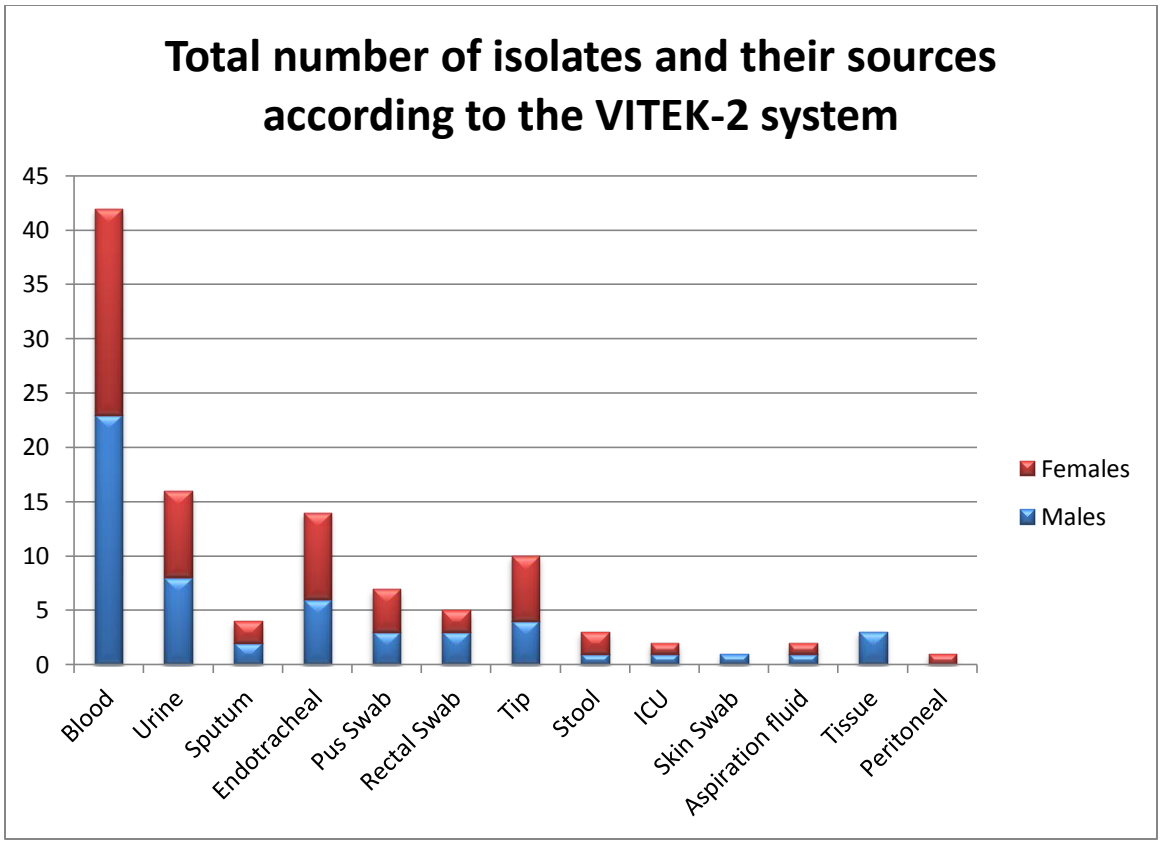


Figure 8. Bar Chart showing the total number of male and female samples isolated and their respective sources identified by VITEK-2 System.

As shown in figure 8. The majority of samples identified as positive for *Acinetobacter baumannii* were collected from blood, totaling 42, or 38.1% of the total positives. This was followed by samples collected from urine, totalling 16, or 16.5% of the total positive. The third most prevalent source was from endotracheal samples, totaling 14, or 12.7% of the total positive sample. The fourth prevalent source was from tip samples, totaling 10, or 9.1%.

3.2.Genotypic Identification

3.2.1. Polymerase chain reaction (PCR)

The purpose of this experiment was to investigate the presence of OXA-69 gene, which signifies the presence or positive identification of *Acinetobacter baumannii*. PCR is a form of genotypic test, which bases its identification on the presence or absence of a specific gene or number of genes inside a bacterium.

3.2.2. PCR Primer data:

The primers used for the PCR reaction was the OXA-69 gene. Primers OXA-69 Forward and OXA-69 Reverse were used for the PCR. The primers and expected fragment size were as shown in the materials and methods.

Upon completion of the VITEK-2 identification, the samples were then further identified using the PCR method by amplifying the OXA-69 gene. The amplification of this gene is a positive genetic indicator of the *Acinetobacter baumannii* species.

Of the 110 samples, only 88 were identified as positive by OXA-69 PCR, suggesting that the VITEK-2 system had an error of 20 %.

The positive isolates were then pictured below in the gels below showing their bands that identify the OXA-69 gene, Figures 9,10,11 for isolates 1 to 88, all showing positive bands at 975bp.

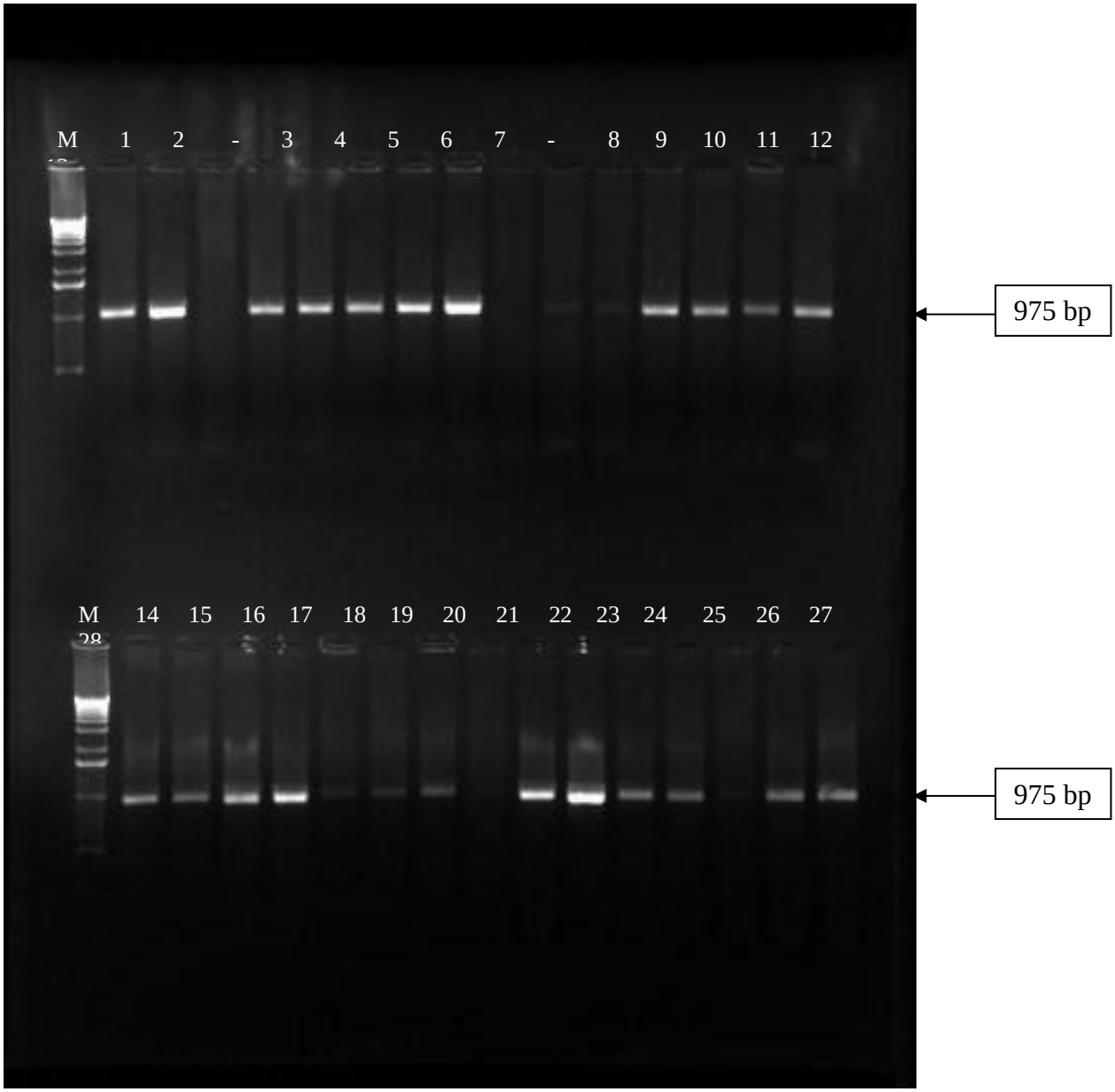


Figure 9. Gel showing *Acinetobacter baumannii* identification using OXA-69 primer for isolates 1 to 28. M= 1kb ladder. Each lane is in accordance to Table 1, whereby each isolate number is recorded above its respective band. Negative lanes 3 and 9 were excluded from the results.

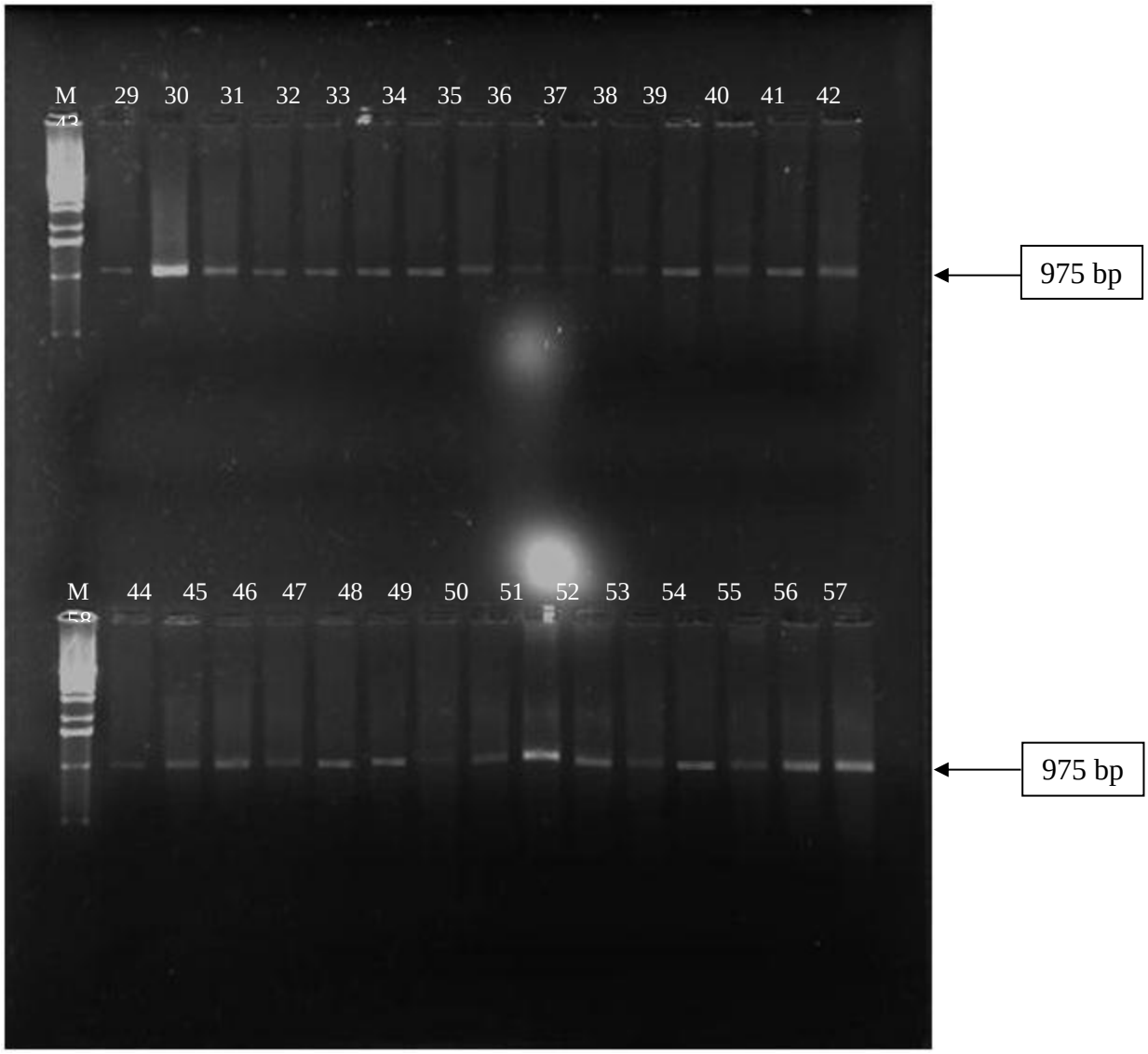


Figure 10. Gel showing *Acinetobacter baumannii* identification using OXA-69 primer for isolates 29 to 58. M= 1kb ladder

All the above samples in the gel were identified as positive, and each isolate number is recorded above its respective lane, and can be referenced with Table 1.

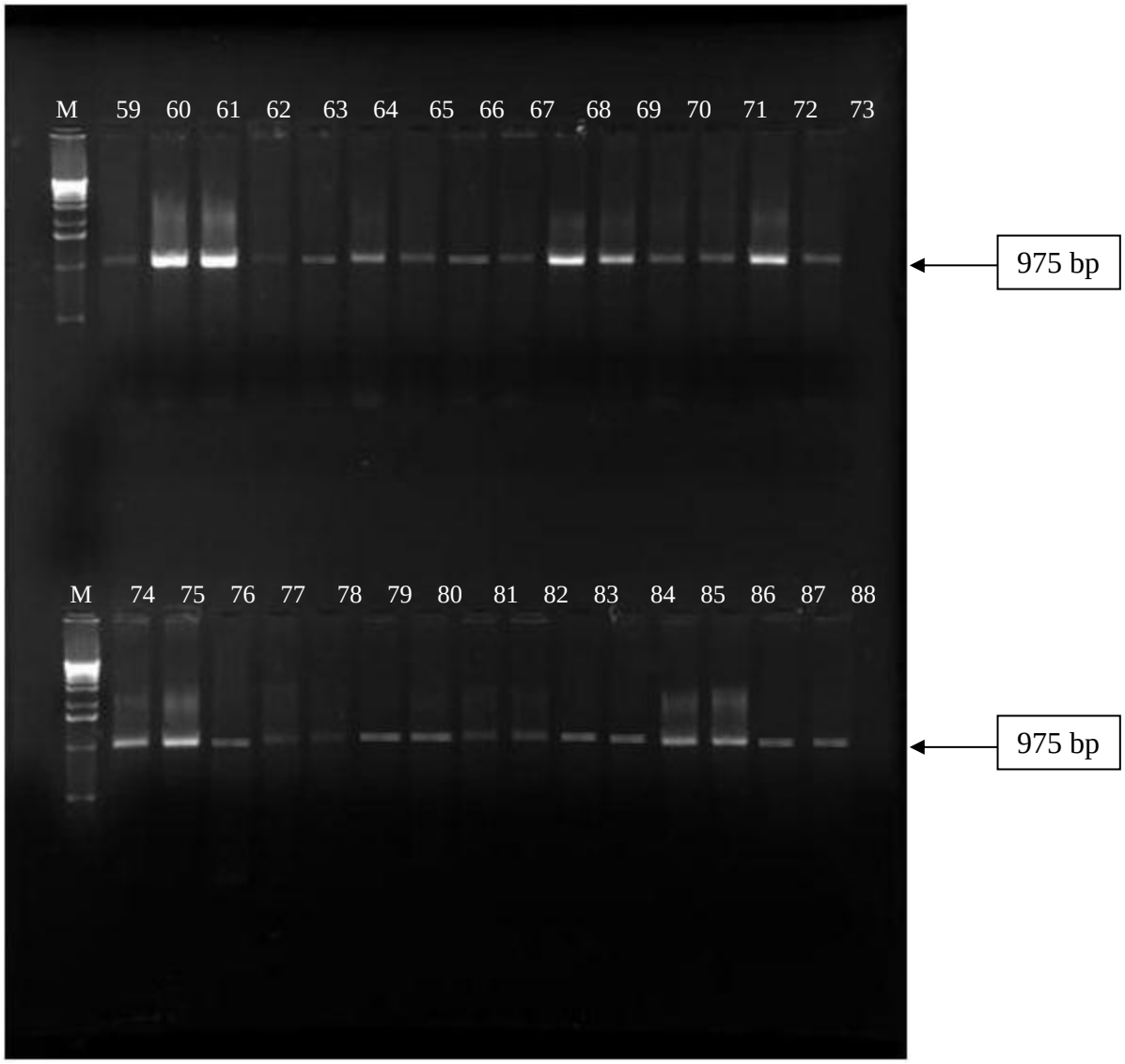


Figure 11. Gel showing *Acinetobacter baumannii* identification using OXA-69 primer for isolates 59 to 88. M= 1kb ladder

All the above samples in the gel were identified as positive, and each isolate number is recorded above its respective lane, and can be referenced with Table 1.

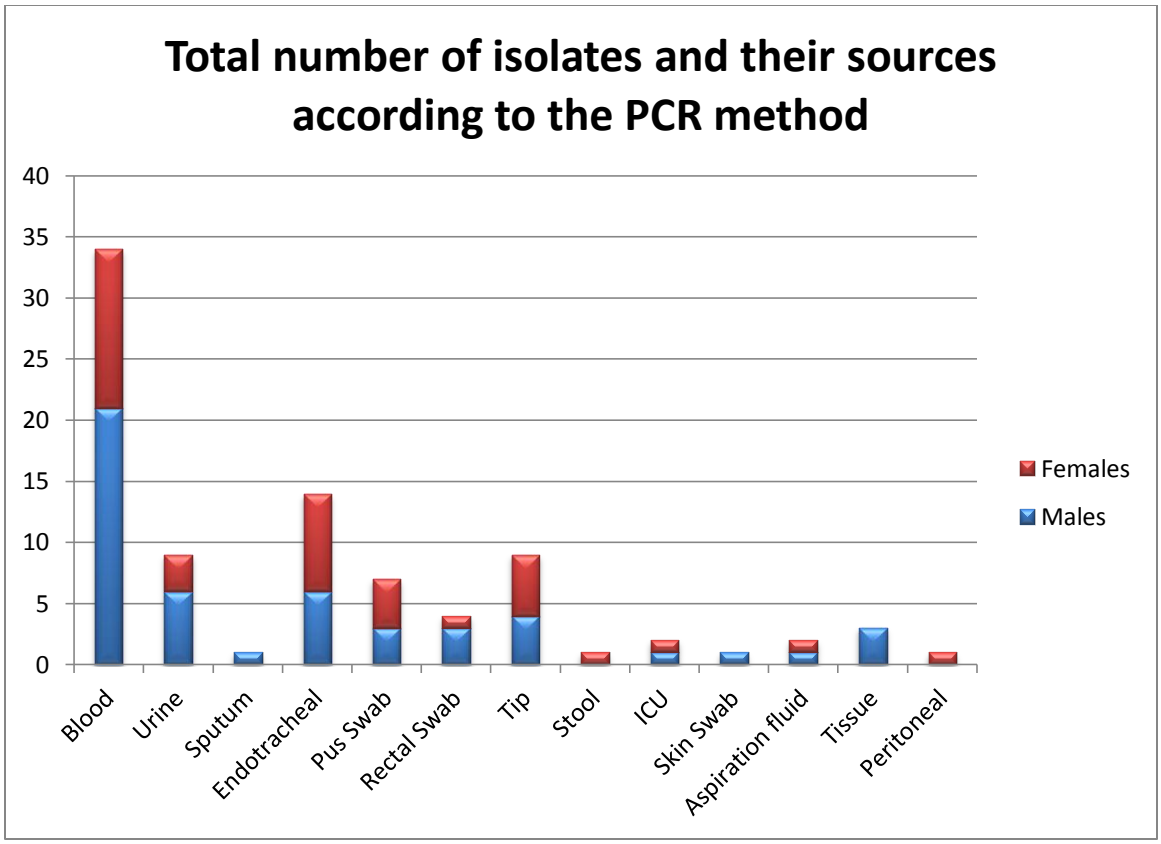


Figure 12. Bar Chart showing the total male and female number of positive samples isolated and their respective sources identified by PCR method.

As shown in figure 12. The majority of samples identified as positive for *Acinetobacter baumannii* were collected from blood, totaling 34, or 38.6% of the total positives. This was followed by samples collected from endotracheal, totaling 14, or 15.9% of the total positive. Both the third and fourth most prevalent sources were from urine and tip samples, totaling 9, or 10.2% of the total positive sample.

3.3.Minimal Inhibitory Concentration (MIC) Test Results

Two of the main carbapenems were used in this study: Meropenem (MEM) and Imipenem (IMP). They are considered as the last source of defence from multi-drug resistant (MDR) strains of *Acinetobacter baumannii* (Peleg *et al*., 2008).

The MIC break points for each sample were recorded under both antibiotic columns (MEM) and (IMP), with the resistance (R), intermediacy (I), and sensitivity (S) identified in accordance to the British Society for Antimicrobial Chemotherapy (BSAC, 2012) version 11.1 (Table 2).

Table 2. Showing MIC Breakpoints for Imipenem and Meropenem.

Antibiotic	**MIC Breakpoint (mg/L)**		
	R>	I	S≤
Imipenem (IMP)	8	4-8	2
Meropenem (MER)	8	4-8	2

The total number of *Acinetobacter baumannii* samples from each year is as follows: 2006 = 26 samples, 2007 = 28 samples and 2008 = 34 samples.

The percentage of isolates resistant to Meropenem was: 23.8%

The percentage of isolates resistant to Imipenem was: 31.8%

The number of Males with isolates resistant to either of the carbapenems was 19, which accounts to 21.5% of the total samples. Whereas the number of females with isolates resistant to either carbapenems was 10, accounting for 11.3% of the total samples.

Overall carbapenem resistance was observed in 55.7% of the total isolates.

While the resistance of Meropenem in each year was as follows: 2006 = 4 samples, (4.5%). 2007 = 7 samples, (7.9%). 2008 = 10 samples, (11%). (Figures 14 and 16)

According to each year, resistance of Imipenem was as follows: 2006 = 7 samples, (7.9%). 2007 = 8 samples, (9%). 2008 = 13 samples, (14.7%). (Figures 15 and 17)

An increase was noticed in the number of resistant bacteria to Imipenem and Meropenem from 2006 to 2008 as shown in Figure 13 below.

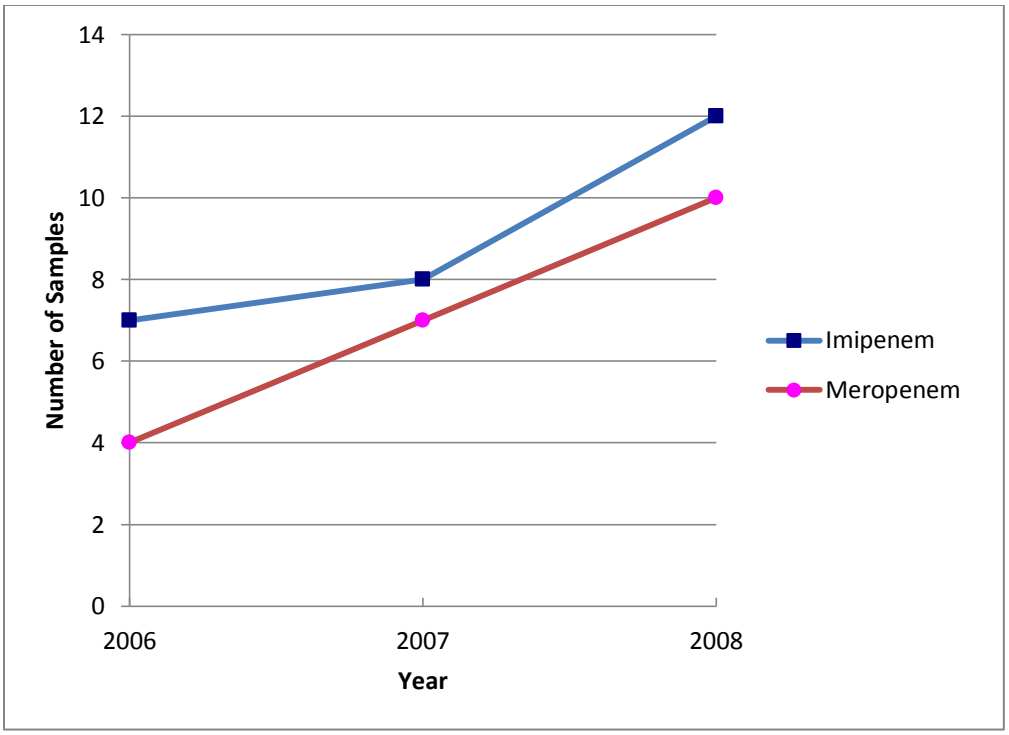


Figure 13. Line Graph showing the steady increase in the number of resistant *Acinetobacter baumannii* samples during the years 2006, 2007, 2008.

Table 3. The total number of Meropenem Sensitive, Intermediate, and Resistant *Acinetobacter baumannii* isolates.

Year	Sensitive	Intermediate	Resistant	Total Number
2006	14	8	4	26
2007	14	7	7	28
2008	13	11	10	34

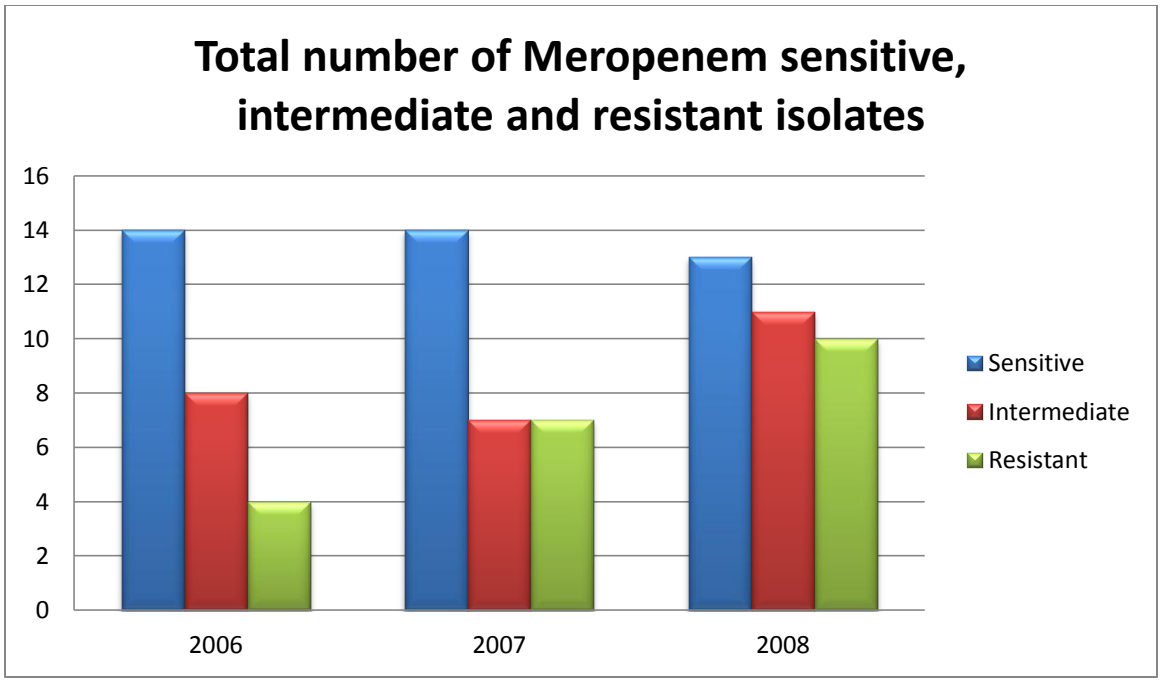


Figure 14. Bar Chart showing the total number of Meropenem Sensitive, Intermediate, and Resistant *Acinetobacter baumannii* isolates.

As shown in table 3 above, the number of Meropenem sensitive, intermediate, and resistant *Acinetobacter baumannii* isolate numbers were all recorded. Figure 14 better describes the table information, showing that there was an increase Meropenem resistance and intermediate numbers over the years. The highest number of Meropenem resistant samples was noticed to be in 2008, also the highest number of Meropenem intermediate samples was also found to be in 2008. The lowest number of Meropenem resistance was found in 2006. This indicates a clear increase in the number of Meropenem resistant samples over the years 2006-2008. There was no significant change in the number of sensitive samples over the 3 years, but a slight decrease in the last year, 2008.

Table 4. The total number of Imipenem Sensitive, Intermediate, and Resistant *Acinetobacter baumannii* isolates.

Year	Sensitive	Intermediate	Resistant	Total Number
2006	16	3	7	26
2007	10	10	8	28
2008	8	13	13	34

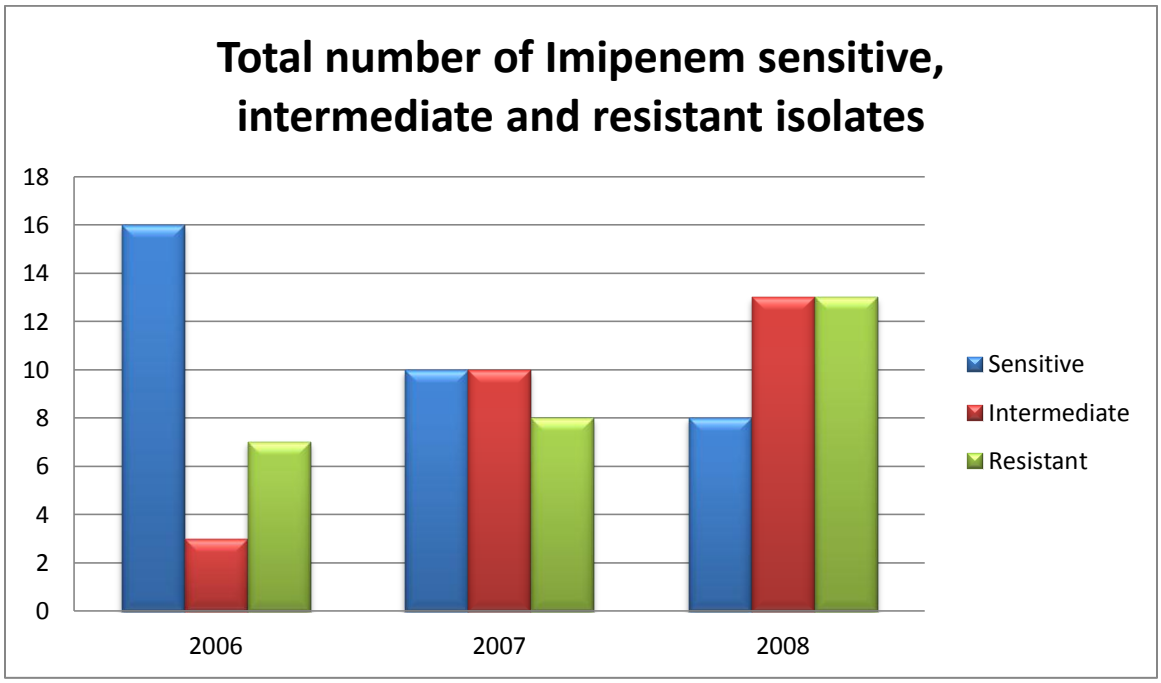


Figure 15. Bar Chart showing the total number of Imipenem Sensitive, Intermediate, and Resistant *Acinetobacter baumannii* isolates.

As shown in table 4 above, the number of Imipenem sensitive, intermediate, and resistant *Acinetobacter baumannii* isolate numbers were all recorded. Figure 15 better describes the table information, showing that an increase in overall resistance to Imipenem was noticed over the years of study. The highest number of Imipenem resistant samples was found in 2008, with also

the highest number of Imipenem intermediate samples in 2008. Conversely, the lowest number of Imipenem resistant samples was found in 2006, with the lowest number of Imipenem intermediate samples also in 2006. This data clearly indicates an increase in the number of Imipenem resistant samples over the years 2006-2008. There was a major decrease in the number of sensitive samples over the 3 years of study.

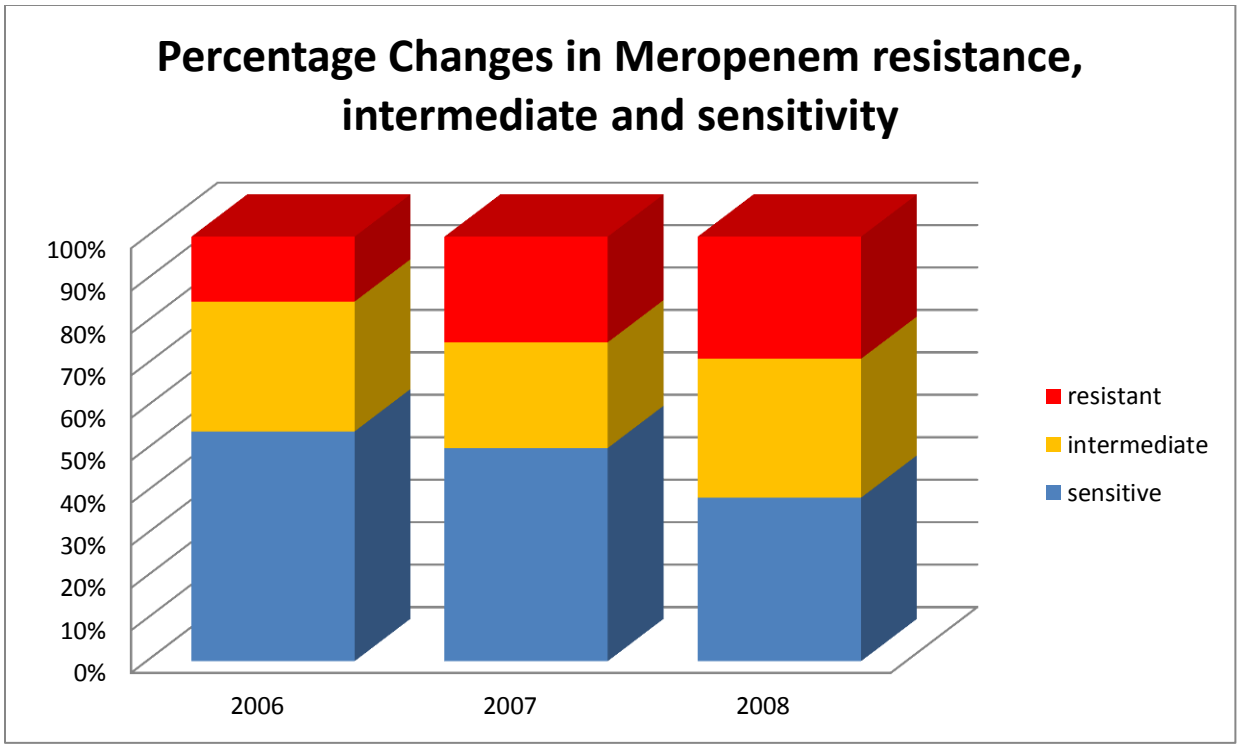


Figure 16. Showing Stacked Column Bar Chart showing the percentage changes in resistance, intermediate, and sensitivity of the *Acinetobacter baumannii* samples to Meropenem, for the years 2006, 2007, 2008.

From the figure 16 above we can clearly see a gradual increase in the overall percentage of Meropenem resistant samples, which are shown in the top red bars, and a gradual decrease in the percentage of Meropenem sensitive samples, what are shown in the bottom blue bars of the stacked column bar chart. The percentage of Meropenem intermediate samples, shown in the middle orange bars, also increased over the 3 years of study.

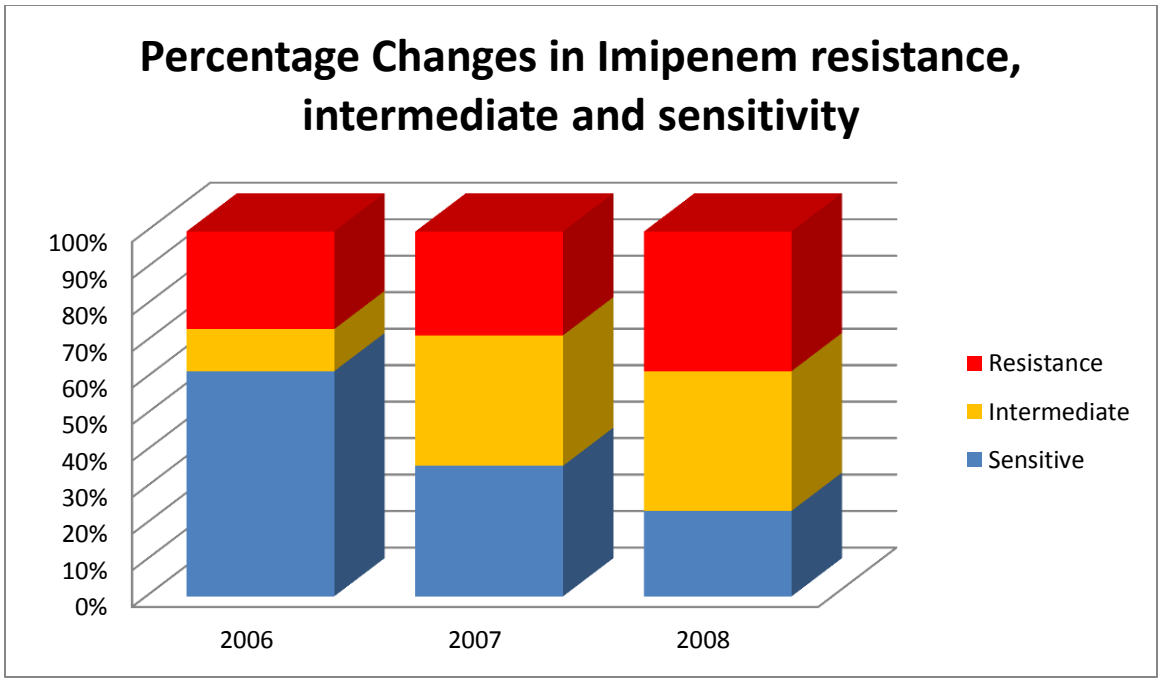


Figure 17. Showing. Stacked Column Bar Chart showing the percentage changes in resistance, intermediate, and sensitivity of the *Acinetobacter baumannii* samples to Imipenem, for the years 2006, 2007, 2008.

From the figure 17 above we can clearly see a slight increase in the overall percentage of Imipenem resistant samples, which are shown in the top red bars, but we noticed a significant decrease in the percentage of Imipenem sensitive samples, that are shown in the bottom blue bars of the stacked column bar chart, from 2006 to 2008. The percentage of Imipenem intermediate samples also significantly increased over the 3 years of study.

Table 5. Shows the samples with their susceptibility to each antibiotic. The sensitive isolates were marked in light blue colour, the intermediate in orange and resistant isolates in red.

Table 5. Minimum Inhibitory Concentration (MIC) Test

Sample No.	Date	Sex	Source	MEM	IMP
1	2006	M	Rectal Swab	0.25	0.5
2	2006	M	ICU	4	1
3	2006	M	Rectal Swab	1	1
4	2006	F	Endotracheal	0.25	0.25
5	2006	M	Urine	4	2
6	2006	M	Tip	0.25	0.5
7	2006	M	Urine	16	16
8	2006	F	Urine	2	2
9	2006	F	Blood	16	16
10	2006	M	Blood	2	2
11	2006	M	Blood	1	2
12	2006	M	Tip	0.5	2
13	2006	M	Blood	8	16
14	2006	M	Blood	8	8
15	2006	M	Blood	8	16
16	2006	M	Urine	0.5	2
17	2006	F	ICU	0.12	0.5
18	2006	F	Blood	4	4
19	2006	M	Blood	4	8
20	2006	M	Endotracheal	8	16
21	2006	F	Blood	16	16
22	2006	M	Blood	16	16
23	2006	F	Tip	0.25	0.25
24	2006	F	Tip	0.25	0.5
25	2006	M	Pus Swab	0.12	0.5
26	2006	M	Tissue	0.25	1
27	2007	M	Tissue	0.25	0.25
28	2007	F	Urine	0.03	0.25
29	2007	F	Exit site Swab	0.06	0.12
30	2007	F	Tip	0.5	0.5
31	2007	M	Blood	8	8
32	2007	M	Blood	2	4
33	2007	M	Blood	16	16
34	2007	M	Blood	8	16
35	2007	M	Skin Swab	8	8

36	2007	M	Blood	16	16
37	2007	M	Blood	8	16
38	2007	F	Blood	4	8
39	2007	F	Endotracheal	2	4
40	2007	M	Endotracheal	0.5	2
41	2007	F	Endotracheal	2	4
42	2007	F	Endotracheal	0.25	1
43	2007	M	Urine	0.12	0.5
44	2007	F	Pus Swab	0.06	0.5
45	2007	M	Endotracheal	1	2
46	2007	F	Endotracheal	8	8
47	2007	M	Sputum	1	4
48	2007	M	Blood	16	32
49	2007	M	Blood	16	16
50	2007	M	Aspiration Fluid	0.5	0.5
51	2007	M	Rectal Swab	8	8
52	2007	M	Pus Swab	16	8
53	2007	F	Pus Swab	16	16
54	2007	F	Pus Swab	16	16
55	2008	M	Blood	16	16
56	2008	M	Endotracheal	0.25	0.5
57	2008	M	Blood	4	16
58	2008	F	Blood	8	4
59	2008	F	Blood	8	8
60	2008	M	Urine	16	16
61	2008	M	Blood	4	8
62	2008	F	Blood	8	16
63	2008	M	Tip	2	4
64	2008	M	Blood	16	16
65	2008	F	Endotracheal	16	16
66	2008	F	Pertonial	0.25	0.25
67	2008	F	Rectal Swab	2	4
68	2008	M	Blood	16	32
69	2008	M	Urine	8	16
70	2008	F	Pus Swab	4	8
71	2008	F	Blood	2	2
72	2008	M	Endotracheal	0.5	0.5
73	2008	M	Tissue	16	32
74	2008	M	Pus Swab	4	4
75	2008	M	Blood	2	4
76	2008	F	Tip	0.015	0.06
77	2008	M	Tip	0.03	0.12
78	2008	M	Endotracheal	2	8
79	2008	F	Urine	2	8
80	2008	F	Blood	4	2

81	2008	F	Endotracheal	8	8
82	2008	F	Blood	2	8
83	2008	F	Blood	16	16
84	2008	F	Blood	4	4
85	2008	F	Endotracheal	16	16
86	2008	F	Tip	16	16
87	2008	F	Aspiration Fluid	0.03	0.25
88	2008	F	Blood	16	32

3.4.Sequencing of PCR products

The DNA Sequencing of all 88 OXA-51-like genes confirmed that of the strains, 50% of them, were found to contain *bla*OXA-64, which is a significant percentage and unprecedented elsewhere. The results are shown in table 7 as well as the figure 20 showing a percentage pie chart of all the isolates in the study.

The first and most prominent OXA-51-like gene found in the samples was the OXA-64, with 50%. The second most prominent was the OXA-98 which was found in 18% of the isolates. OXA-66 was third down the percentage list and was found in 10% of the isolates. OXA-69 and OXA-71 were both found in 7% of the isolates. OXA-51 was found in 3% of the isolates. OXA-68 was found in 2% of the isolates, and finally OXA-70 and OXA-117 were found in 1% of the isolates.

All the sequences were cross-checked with the GenBank database before their identification, any sequences that were not found in the GenBank were sequenced again to double check for mistakes, before being sent to the GenBank database and get published as a newly found OXA nucleotide sequence. In this study a new and complete nucleotide sequence for the OXA-117 enzyme was found, the complete 825 bp sequence data of OXA-117 was then deposited in the GenBank nucleotide database under accession number: GQ423625.1

The majority of the OXA-64 containing *Acinetobacter baumannii* were isolated from blood. Whereas the rest of the OXA-51-like genes were not found to be particularly associated with any specific source of infection. The samples were varied in their collection source, varying from blood to urine, skin to sputum.

Table 6. OXA-51-like types

Sample No.	Serial No.	Date	Sex	Source	OXA-51-like types
1	M16	2006	M	Rectal Swab	64
2	M17	2006	M	ICU	64
3	M20	2006	M	Rectal Swab	64
4	M21	2006	F	Endotracheal	64
5	M28	2006	M	Urine	64
6	M35	2006	M	Tip	69
7	M36	2006	M	Urine	117
8	M37	2006	F	Urine	69
9	M48	2006	F	Blood	64
10	M49	2006	M	Blood	64
11	M50	2006	M	Blood	64
12	M51	2006	M	Tip	64
13	M53	2006	M	Blood	64
14	M54	2006	M	Blood	64
15	M55	2006	M	Blood	64
16	M56	2006	M	Urine	64
17	M57	2006	F	ICU	51

18	M62	2006	F	Blood	98
19	M65	2006	M	Blood	64
20	M66	2006	M	Endotracheal	66
21	M67	2006	F	Blood	64
22	M68	2006	M	Blood	64
23	M71	2006	F	Tip	64
24	M72	2006	F	Tip	64
25	M73	2006	M	Pus Swab	98
26	M74	2006	M	Tissue	98
27	M75	2007	M	Tissue	98
28	M79	2007	F	Urine	64
29	M80	2007	F	Rectal Swab	64
30	M84	2007	F	Tip	66
31	M85	2007	M	Blood	64
32	M86	2007	M	Blood	64
33	M87	2007	M	Blood	64
34	M88	2007	M	Blood	64
35	M89	2007	M	Skin Swab	64
36	M96	2007	M	Blood	64
37	M97	2007	M	Blood	64
38	M100	2007	F	Blood	64
39	M106	2007	F	Endotracheal	69
40	M110	2007	M	Endotracheal	69
41	M113	2007	F	Endotracheal	69
42	M114	2007	F	Endotracheal	69
43	M123	2007	M	Urine	64
44	M140	2007	F	Pus Swab	98
45	M158	2007	M	Endotracheal	71
46	M159	2007	F	Endotracheal	70
47	M160	2007	M	Sputum	66
48	M161	2007	M	Blood	98
49	M165	2007	M	Blood	98
50	M167	2007	M	Aspiration Fluid	64
51	M169	2007	M	Exit site Swab	98
52	M178	2007	M	Pus Swab	64
53	M180	2007	F	Pus Swab	98
54	M184	2007	F	Pus Swab	98
55	K1	2008	M	Blood	64
56	K2	2008	M	Endotracheal	64
57	K5	2008	M	Blood	98
58	K6	2008	F	Blood	98
59	K7	2008	F	Blood	66
60	K8	2008	M	Urine	98
61	K10	2008	M	Blood	71

62	K11	2008	F	Blood	66
63	K12	2008	M	Tip	98
64	K13	2008	M	Blood	68
65	K15	2008	F	Endotracheal	66
66	K16	2008	F	Pertonial	64
67	K17	2008	F	Rectal Swab	64
68	K18	2008	M	Blood	66
69	K19	2008	M	Urine	98
70	K21	2008	F	Pus Swab	106
71	K24	2008	F	Blood	98
72	K25	2008	M	Endotracheal	64
73	K26	2008	M	Tissue	68
74	K27	2008	M	Pus Swab	71
75	K29	2008	M	Blood	51
76	K30	2008	F	Tip	66
77	K39	2008	M	Tip	66
78	K44	2008	M	Endotracheal	64
79	K45	2008	F	Urine	64
80	K47	2008	F	Blood	64
81	K48	2008	F	Endotracheal	64
82	K88	2008	F	Blood	64
83	K89	2008	F	Blood	64
84	K90	2008	F	Blood	71
85	K93	2008	F	Endotracheal	71
86	K94	2008	F	Tip	64
87	K95	2008	F	Aspiration Fluid	71
88	K97	2008	F	Blood	64

The total number of OXA samples that were sequenced was 88. The number of each sequence type is shown in table 7 below. OXA-64 was found in the majority of the samples, in 44 samples. OXA-98 was the second most prevalent, found in 16 samples, and OXA-66 was third most prevalent, found in 9 samples. This study was the first to show the OXA-type distribution for *Acinetobacter baumannii* in Kuwait. In total 10 OXA-types were discovered in the samples isolated from the Mubarak Al-Kabeer hospital.

Sequence Groups 1,2,3 and 4 were all identified in this study, in accordance to Turton *et al*, (2007). See table 7.

Table 7. Showing all the OXA-types identified in the study, their total number from the samples, and the identified sequence groups they represent.

OXA-TYPE	Number of samples	Sequence Group
OXA-64	44	-
OXA-69	6	2
OXA-117	1	-
OXA-51	2	4
OXA-98	16	-
OXA-66	9	1
OXA-71	6	3
OXA-70	1	-
OXA-68	2	-
OXA-106	1	-

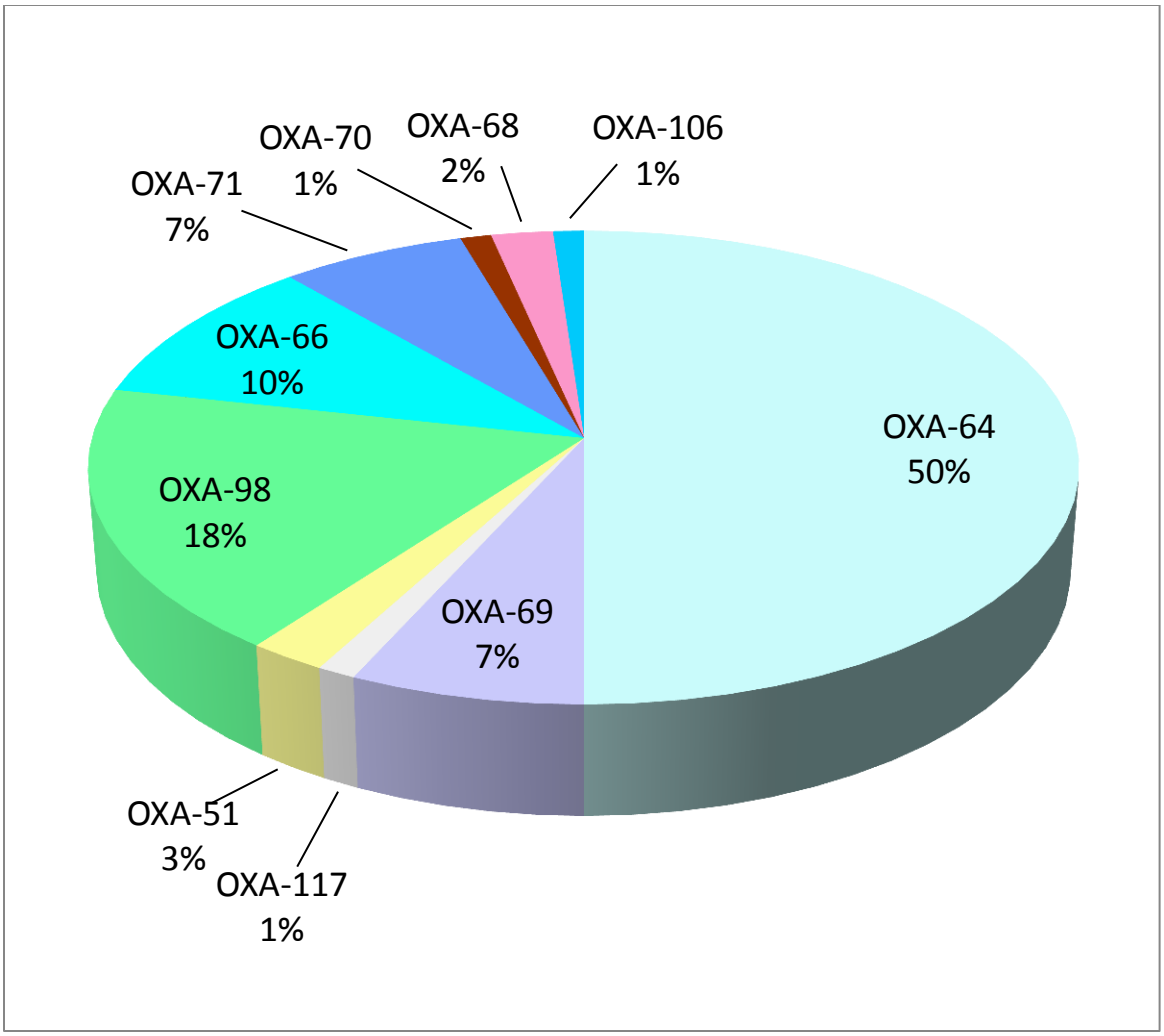


Figure 18. Pie Chart showing the distribution of all the *bla*$_{\text{OXA-51-like}}$ genes identified in Mubarak Al-Kabeer Hospital in Kuwait.

From the pie chart in Figure 18 above, it can be clearly seen that the majority of the strains contained the OXA-64 enzyme, with 50% accounting for them. Next the OXA-98 which was found in 18% of the total samples, and followed by OXA-66 which was found in 10% of the total *Acinetobacter baumannii* isolates. Both OXA-71 and OXA-69 were found in 7% of the total isolates. Table 6 was used to calculate the data in this pie chart.

3.5.Nucleotide Sequence Result Comparisons

Below is the series of Nucleotide Sequence results which were identified, and each compared to the OXA-64 sequence, because it was more prevalent in this study.

The highlighted nucleotides represent the differences between each respective OXA in each analysis.

3.5.1. OXA-64 and OXA-71

```
OXA-64 1    ATGAACATTAAAGCACTCTTACTTATAACAAGCGCTATTTTTATTTCAGCCTGCTCACCT  60
            ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
OXA-71 1    ATGAACATTAAAGCCCTCTTACTTATAACAAGCGCTATTTTTATTTCAGCCTGCTCACCT  60

OXA-64 61   TATATAGTGACTGCTAATCCAAATCACAGCGCTTCAAAATCTGATGAAAAAGGAGAGAAA  120
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
OXA-71 61   TATATAGTGACTGCTAATCCAAATCACAGCGCTTCAAAATCTGATGAAAAAGCAGAGAAA  120

OXA-64 121  ATTAAAAATTTATTTAACGAAGCACACACTACGGGTGTTTTAGTTATCCAACAAGGCCAA  180
            |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
OXA-71 121  ATTAAAAATTTATTTAACGAAGCACACACTACGGGTGTCTTAGTTATCCAACAAGGCCAA  180

OXA-64 181  ACTCAACAAAGCTATGGTAATGATCTTGCTCGTGCTTCGACCGAGTATGTACCTGCTTCG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-71 181  ACTCAACAAAGCTATGGTAATGATCTTGCTCGTGCTTCGACCGAGTATGTACCTGCTTCG  240

OXA-64 241  ACCTTCAAAATGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAACCACCACAGAA  300
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
OXA-71 241  ACCTTCAAAATGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGACAACCACCACAGAA  300

OXA-64 301  GTATTTAAGTGGGACGGGCAAAAAAGGCTATTCCCAGAATGGGAAAAGGACATGACCCTA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-71 301  GTATTTAAGTGGGACGGGCAAAAAAGGCTATTCCCAGAATGGGAAAAGGACATGACCCTA  360

OXA-64 361  GGCGATGCTATGAAAGCTTCCGCTATTCCGGTTTATCAAGATTTAGCTCGTCGTATTGGA  420
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-71 361  GGCGACGCTATGAAAGCTTCCGCTATTCCGGTTTATCAAGATTTAGCTCGTCGTATTGGA  420

OXA-64 421  CTTGAACTCATGTCTAAGGAAGTGAAGCGTGTTGGTTATGGCAATGCAGATATCGGTACC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-71 421  CTTGAACTCATGTCTAAGGAAGTGAAGCGTGTTGGTTATGGCAATGCAGATATCGGTACC  480

OXA-64 481  CAAGTCGATAATTTTTGGCTGGTGGGTCCTTTAAAAATTACTCCTCAGCAAGAGGCACAG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-71 481  CAAGTCGATAATTTTTGGCTGGTGGGTCCTTTAAAAATTACTCCTCAGCAAGAGGCACAA  540

OXA-64 541  TTTGCTTACAAGCTAGCTAATAAAACGCTTCCATTTAGCCCAAAAGTCCAAGATGAAGTG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-71 541  TTTGCTTACAAGCTAGCTAATAAAACGCTTCCATTTAGCCCAAAAGTCCAAGATGAAGTG  600

OXA-64 601  CAATCCATGTTATTCATAGAAGAAAAGAATGGAAATAAAATATACGCAAAAAGTGGTTGG  660
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
OXA-71 601  CAATCCATGCTATTCATAGAAGAAAAGAATGGAAATAAAATATACGCAAAAAGTGGTTGG  660
```

```
OXA-64 661  GGATGGGATGTAGACCCACAAGTAGGCTGGTTAACTGGATGGGTTGTTCAGCCTCAAGGG  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-71 661  GGATGGGATGTAGACCCACAAGTAGGCTGGTTAACTGGATGGGTTGTTCAGCCTCAAGGA  720

OXA-64 721  AATATTGTAGCGTTCTCCCTTAACTTAGAAATGAAAAAAGGAATACCTAGCTCTGTTCGA  780
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
OXA-71 721  AATATTGTAGCATTCTCCCTTAACTTAGAAATGAAAAAAGGAATACCTAGCTCTGTTCGA  780

OXA-64 781  AAAGAGATTACTTATAAAAGTTTAGAACAATTAGGTATTTTATAG  825
            |||||||||||||||||||||||||||||||||||||||||||||
OXA-71 781  AAAGAGATTACTTATAAAAGTTTAGAACAATTAGGTATTTTATAG  825
```

3.5.2. OXA-64 and OXA-66

```
OXA-64 1    ATGAACATTAAAGCACTCTTACTTATAACAAGCGCTATTTTTATTTCAGCCTGCTCACCT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-66 1    ATGAACATTAAAGCACTCTTACTTATAACAAGCGCTATTTTTATTTCAGCCTGCTCACCT  60

OXA-64 61   TATATAGTGACTGCTAATCCAAATCACAGCGCTTCAAAATCTGATGAAAAAGGAGAGAAA  120
            ||||||||||||||||||||||||||||||||||||||||||||| ||||| |||||||
OXA-66 61   TATATAGTGACTGCTAATCCAAATCACAGCGCTTCAAAATCTGATGTAAAAGCAGAGAAA  120

OXA-64 121  ATTAAAAATTTATTTAACGAAGCACACACTACGGGTGTTTTAGTTATCCAACAAGGCCAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-66 121  ATTAAAAATTTATTTAACGAAGCACACACTACGGGTGTTTTAGTTATCCAACAAGGCCAA  180

OXA-64 181  ACTCAACAAAGCTATGGTAATGATCTTGCTCGTGCTTCGACCGAGTATGTACCTGCTTCG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-66 181  ACTCAACAAAGCTATGGTAATGATCTTGCTCGTGCTTCGACCGAGTATGTACCTGCTTCG  240

OXA-64 241  ACCTTCAAAATGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAACCACCACAGAA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-66 241  ACCTTCAAAATGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAACCACCACAGAA  300

OXA-64 301  GTATTTAAGTGGGACGGGCAAAAAAGGCTATTCCCAGAATGGGAAAAGGACATGACCCTA  360
            |||||||||||||| ||  |||||||| ||||||||||||||||||||||||||||||||
OXA-66 301  GTATTTAAGTGGGATGGTAAAAAAAGGTTATTCCCAGAATGGGAAAAGGACATGACCCTA  360

OXA-64 361  GGCGATGCTATGAAAGCTTCCGCTATTCCGGTTTATCAAGATTTAGCTCGTCGTATTGGA  420
            |||||||| |||||||||||||||||||| ||||||||||||||||||||||||||||||
OXA-66 361  GGCGATGCCATGAAAGCTTCCGCTATTCCAGTTTATCAAGATTTAGCTCGTCGTATTGGA  420

OXA-64 421  CTTGAACTCATGTCTAAGGAAGTGAAGCGTGTTGGTTATGGCAATGCAGATATCGGTACC  480
            |||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-66 421  CTTGAGCTCATGTCTAAGGAAGTGAAGCGTGTTGGTTATGGCAATGCAGATATCGGTACC  480

OXA-64 481  CAAGTCGATAATTTTTGGCTGGTGGGTCCTTTAAAAATTACTCCTCAGCAAGAGGCACAG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-66 481  CAAGTCGATAATTTTTGGCTGGTGGGTCCTTTAAAAATTACTCCTCAGCAAGAGGCACAG  540

OXA-64 541  TTTGCTTACAAGCTAGCTAATAAAACGCTTCCATTTAGCCCAAAAGTCCAAGATGAAGTG  600
            |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
OXA-66 541  TTTGCTTACAAGCTAGCTAATAAAACGCTTCCATTTAGCCAAAAAGTCCAAGATGAAGTG  600

OXA-64 601  CAATCCATGTTATTCATAGAAGAAAAGAATGGAAATAAAATATACGCAAAAAGTGGTTGG  660
            |||||||| |||||||||||||||||||||||| |||||||||||||||||||||||||||
OXA-66 601  CAATCCATGCTATTCATAGAAGAAAAGAATGGAAACAAAATATACGCAAAAAGTGGTTGG  660

OXA-64 661  GGATGGGATGTAGACCCACAAGTAGGCTGGTTAACTGGATGGGTTGTTCAGCCTCAAGGG  720
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
OXA-66 661  GGATGGGATGTAACCCACAAGTAGGCTGGTTAACTGGATGGGTTGTTCAGCCTCAAGGG  720

OXA-64 721  AATATTGTAGCGTTCTCCCTTAACTTAGAAATGAAAAAAGGAATACCTAGCTCTGTTCGA  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-66 721  AATATTGTAGCGTTCTCCCTTAACTTAGAAATGAAAAAAGGAATACCTAGCTCTGTTCGA  780

OXA-64 781  AAAGAGATTACTTATAAAAGTTTAGAACAATTAGGTATTTTATAG  825
            |||||||||||||||||||| |||||||||||||||||||||||
OXA-66 781  AAAGAGATTACTTATAAAAGCTTAGAACAATTAGGTATTTTATAG  825
```

3.5.3. OXA-64 and OXA-69

```
OXA-64 1    ATGAACATTAAAGCACTCTTACTTATAACAAGCGCTATTTTTATTTCAGCCTGCTCACCT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-69 1    ATGAACATTAAAGCACTCTTACTTATAACAAGCGCTATTTTTATTTCAGCCTGCTCACCT  60

OXA-64 61   TATATAGTGACTGCTAATCCAAATCACAGCGCTTCAAAATCTGATGAAAAAGGAGAGAAA  120
            ||||||||||||||||||||||||||||| |||||||||||||||| |||| ||||||||
OXA-69 61   TATATAGTGACTGCTAATCCAAATCACAGTGCTTCAAAATCTGATGACAAAGCAGAGAAA  120

OXA-64 121  ATTAAAAATTTATTTAACGAAGCACACACTACGGGTGTTTTAGTTATCCAACAAGGCCAA  180
            |||||||||||||||||||||||||||||||||||||||||||||||| ||||| |||
OXA-69 121  ATTAAAAATTTATTTAACGAAGCACACACTACGGGTGTTTTAGTTATCCATCAAGGTCAA  180

OXA-64 181  ACTCAACAAAGCTATGGTAATGATCTTGCTCGTGCTTCGACCGAGTATGTACCTGCTTCG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-69 181  ACTCAACAAAGCTATGGTAATGATCTTGCTCGTGCTTCGACCGAGTATGTACCTGCTTCG  240

OXA-64 241  ACCTTCAAAATGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAACCACCACAGAA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-69 241  ACCTTCAAAATGCTTAATGCTTTGATCGGCCTTGAGCACCATAAGGCAACCACCACAGAA  300

OXA-64 301  GTATTTAAGTGGGACGGGCAAAAAAGGCTATTCCCAGAATGGGAAAAGGACATGACCCTA  360
            |||||||| ||||| ||| ||||||||||||||||||||||||||||| ||||||||||||
OXA-69 301  GTATTTAAATGGGATGGGGAAAAAAGGCTATTCCCAGAATGGGAAAAGAACATGACCCTA  360

OXA-64 361  GGCGATGCTATGAAAGCTTCCGCTATTCCGGTTTATCAAGATTTAGCTCGTCGTATTGGA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-69 361  GGCGATGCTATGAAAGCTTCCGCTATTCCGGTTTATCAAGATTTAGCTCGTCGTATTGGA  420

OXA-64 421  CTTGAACTCATGTCTAAGGAAGTGAAGCGTGTTGGTTATGGCAATGCAGATATCGGTACC  480
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-69 421  CTTGAGCTCATGTCTAAGGAAGTGAAGCGTGTTGGTTATGGCAATGCAGATATCGGTACC  480

OXA-64 481  CAAGTCGATAATTTTTGGCTGGTGGGTCCTTTAAAAATTACTCCTCAGCAAGAGGCACAG  540
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
OXA-69 481  CAAGTCGATAATTTTTGGCTGGTGGGTCCTCTAAAAATTACTCCTCAGCAAGAGGCACAG  540

OXA-64 541  TTTGCTTACAAGCTAGCTAATAAAACGCTTCCATTTAGCCCAAAAGTCCAAGATGAAGTG  600
            |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
OXA-69 541  TTTGCTTACAAGCTAGCTAATAAAACGCTTCCATTTAGCCAAAAAGTCCAAGATGAAGTG  600

OXA-64 601  CAATCCATGTTATTCATAGAAGAAAAGAATGGAAATAAAATATACGCAAAAAGTGGTTGG  660
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-69 601  CAATCCATGCTATTCATAGAAGAAAAGAATGGAAATAAAATATACGCAAAAAGTGGTTGG  660

OXA-64 661  GGATGGGATGTAGACCCACAAGTAGGCTGGTTAACTGGATGGGTTGTTCAGCCTCAAGGG  720
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
OXA-69 661  GGATGGGATGTAAACCCACAAGTAGGCTGGTTAACTGGATGGGTTGTTCAGCCTCAAGGG  720

OXA-64 721  AATATTGTAGCGTTCTCCCTTAACTTAGAAATGAAAAAAGGAATACCTAGCTCTGTTCGA  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OXA-69 721  AATATTGTAGCGTTCTCCCTTAACTTAGAAATGAAAAAAGGAATACCTAGCTCTGTTCGA  780

OXA-64 781  AAAGAGATTACTTATAAAAGTTTAGAACAATTAGGTATTTTATAG  825
            |||||||||||||||||||||||||||||||||||||||||||||
OXA-69 781  AAAGAGATTACTTATAAAAGTTTAGAACAATTAGGTATTTTATAG  825
```

3.6.Protein sequence comparison

Below is the series of Protein Sequence results which were identified, and each compared to the OXA-64 sequence, because it was more prevalent in this study.

The highlighted amino acids represent the differences between each of the respective OXA types in this analysis.

3.6.1. OXA-64 and OXA-71

```
OXA-64 1    MNIKALLLITSAIFISACSPYIVTANPNHSASKSDEKGEKIKNLFNEAHTTGVLVIQQGQ  60
            MNIKALLLITSAIFISACSPYIVTANPNHSASKSDEK EKIKNLFNEAHTTGVLVIQQGQ
OXA-71 1    MNIKALLLITSAIFISACSPYIVTANPNHSASKSDEKAEKIKNLFNEAHTTGVLVIQQGQ  60

OXA-64 61   TQQSYGNDLARASTEYVPASTFKMLNALIGLEHHKATTTEVFKWDGQKRLFPEWEKDMTL  120
            TQQSYGNDLARASTEYVPASTFKMLNALIGLEHHK TTTEVFKWDGQKRLFPEWEKDMTL
OXA-71 61   TQQSYGNDLARASTEYVPASTFKMLNALIGLEHHKTTTTEVFKWDGQKRLFPEWEKDMTL  120

OXA-64 121  GDAMKASAIPVYQDLARRIGLELMSKEVKRVGYGNADIGTQVDNFWLVGPLKITPQQEAQ  180
            GDAMKASAIPVYQDLARRIGLELMSKEVKRVGYGNADIGTQVDNFWLVGPLKITPQQEAQ
OXA-71 121  GDAMKASAIPVYQDLARRIGLELMSKEVKRVGYGNADIGTQVDNFWLVGPLKITPQQEAQ  180

OXA-64 181  FAYKLANKTLPFSPKVQDEVQSMLFIEEKNGNKIYAKSGWGWDVDPQVGWLTGWVVQPQG  240
            FAYKLANKTLPFSPKVQDEVQSMLFIEEKNGNKIYAKSGWGWDVDPQVGWLTGWVVQPQG
OXA-71 181  FAYKLANKTLPFSPKVQDEVQSMLFIEEKNGNKIYAKSGWGWDVDPQVGWLTGWVVQPQG  240

OXA-64 241  NIVAFSLNLEMKKGIPSSVRKEITYKSLEQLGIL  274
            NIVAFSLNLEMKKGIPSSVRKEITYKSLEQLGIL
OXA-71 241  NIVAFSLNLEMKKGIPSSVRKEITYKSLEQLGIL  274
```

3.6.2. OXA-64 and OXA-66

```
OXA-64 1    MNIKALLLITSAIFISACSPYIVTANPNHSASKSDEKGEKIKNLFNEAHTTGVLVIQQGQ  60
            MNIKALLLITSAIFISACSPYIVTANPNHSASKSD K EKIKNLFNEAHTTGVLVIQQGQ
OXA-66 1    MNIKALLLITSAIFISACSPYIVTANPNHSASKSDVKAEKIKNLFNEAHTTGVLVIQQGQ  60

OXA-64 61   TQQSYGNDLARASTEYVPASTFKMLNALIGLEHHKATTTEVFKWDGQKRLFPEWEKDMTL  120
            TQQSYGNDLARASTEYVPASTFKMLNALIGLEHHKATTTEVFKWDG+KRLFPEWEKDMTL
OXA-66 61   TQQSYGNDLARASTEYVPASTFKMLNALIGLEHHKATTTEVFKWDGKKRLFPEWEKDMTL  120

OXA-64 121  GDAMKASAIPVYQDLARRIGLELMSKEVKRVGYGNADIGTQVDNFWLVGPLKITPQQEAQ  180
            GDAMKASAIPVYQDLARRIGLELMSKEVKRVGYGNADIGTQVDNFWLVGPLKITPQQEAQ
OXA-66 121  GDAMKASAIPVYQDLARRIGLELMSKEVKRVGYGNADIGTQVDNFWLVGPLKITPQQEAQ  180

OXA-64 181  FAYKLANKTLPFSPKVQDEVQSMLFIEEKNGNKIYAKSGWGWDVDPQVGWLTGWVVQPQG  240
            FAYKLANKTLPFS KVQDEVQSMLFIEEKNGNKIYAKSGWGWDV+PQVGWLTGWVVQPQG
OXA-66 181  FAYKLANKTLPFSQKVQDEVQSMLFIEEKNGNKIYAKSGWGWDVNPQVGWLTGWVVQPQG  240

OXA-64 241  NIVAFSLNLEMKKGIPSSVRKEITYKSLEQLGIL  274
            NIVAFSLNLEMKKGIPSSVRKEITYKSLEQLGIL
OXA-66 241  NIVAFSLNLEMKKGIPSSVRKEITYKSLEQLGIL  274
```

3.6.3. OXA-64 and OXA-69

```
OXA-64 1    MNIKALLLITSAIFISACSPYIVTANPNHSASKSDEKGEKIKNLFNEAHTTGVLVIQQGQ  60
            MNIKALLLITSAIFISACSPYIVTANPNHSASKSD+K EKIKNLFNEAHTTGVLVI QGQ
OXA-69 1    MNIKALLLITSAIFISACSPYIVTANPNHSASKSDDKAEKIKNLFNEAHTTGVLVIHQGQ  60

OXA-64 61   TQQSYGNDLARASTEYVPASTFKMLNALIGLEHHKATTTEVFKWDGQKRLFPEWEKDMTL  120
            TQQSYGNDLARASTEYVPASTFKMLNALIGLEHHKATTTEVFKWDG+KRLFPEWEK+MTL
OXA-69 61   TQQSYGNDLARASTEYVPASTFKMLNALIGLEHHKATTTEVFKWDGEKRLFPEWEKNMTL  120

OXA-64 121  GDAMKASAIPVYQDLARRIGLELMSKEVKRVGYGNADIGTQVDNFWLVGPLKITPQQEAQ  180
            GDAMKASAIPVYQDLARRIGLELMSKEVKRVGYGNADIGTQVDNFWLVGPLKITPQQEAQ
OXA-69 121  GDAMKASAIPVYQDLARRIGLELMSKEVKRVGYGNADIGTQVDNFWLVGPLKITPQQEAQ  180

OXA-64 181  FAYKLANKTLPFSPKVQDEVQSMLFIEEKNGNKIYAKSGWGWDVDPQVGWLTGWVVQPQG  240
            FAYKLANKTLPFS KVQDEVQSMLFIEEKNGNKIYAKSGWGWDV+PQVGWLTGWVVQPQG
OXA-69 181  FAYKLANKTLPFSQKVQDEVQSMLFIEEKNGNKIYAKSGWGWDVNPQVGWLTGWVVQPQG  240

OXA-64 241  NIVAFSLNLEMKKGIPSSVRKEITYKSLEQLGIL  274
            NIVAFSLNLEMKKGIPSSVRKEITYKSLEQLGIL
OXA-69 241  NIVAFSLNLEMKKGIPSSVRKEITYKSLEQLGIL  274
```

3.7.Complete Nucleotide and Protein sequence of novel OXA-117

This study was able to uncover the complete gene sequence of the OXA-117 gene. The sequence was submitted to GenBank and accepted under accession number: GQ423625

The complete sequence from the start codon to the end codon in shown below:

Start codon →

```
atgaacttaaagcactcttacttataacaagcgctatttttatttcagcctgctcaccttatatagtgtctgctaa
tccaaatcacagtgcttcaaaatctgatgaaaaagcagagaaaattaaaaatttatttaacgaagcacacactacgg
gtgttttagttatccaacaaggccaaactcaacaaagctatggtaatgatcttgctcgtgcttcgaccgagtatgta
cctgcttcgaccttcaaaatgcttaatgctttgatcggccttgagcaccataaggcaaccactacagaagtatttaa
gtgggacgggcaaaaaaggctattcccagaatgggaaaagaacatgaccctaggcgatgctatgaaagcttccgcta
ttccggtttatcaagatttagctcgtcgtattggacttgaactcatgtctaatgaagtgaagcgtgttggttatggc
aatgcagatatcggtacccaagtcgataatttttggctagtgggtcctttaaaaattactcctcagcaagaggcaca
atttgcttacaagctagctaataaaacgcttccatttagccaaaaagtccaagatgaagtgcaatccatgctattca
tagaagaaaagaatggaaataaaatatacgcaaaaagtggttggggatgggatgtaaacccacaagtaggctggtta
actgaatgggttgttcagcctcaagggaatattgtagcgttctcccttaacttagaaatgaaaaaaggaatacctag
ctctgttcgaaaagagattacttataaaagcttagaacaattaggtattttatag
```

← End codon

The complete protein sequence of the novel enzyme OXA-117 is shown below:

```
MNIKALLLITSAIFISACSPYIVSANPNHSASKSDEKAEKIKNLFNEAHTTGVLVIQQGQTQQS
YGNDLARASTEYVPASTFKMLNALIGLEHHKATTTEVFKWDGQKRLFPEWEKNMTLGDAMKASA
IPVYQDLARRIGLELMSNEVKRVGYGNADIGTQVDNFWLVGPLKITPQQEAQFAYKLANKTLPF
SQKVQDEVQSMLFIEEKNGNKIYAKSGWGWDVNPQVGWLTEWVVQPQGNIVAFSLNLEMKKGIP
SSVRKEITYKSLEQLGIL
```

3.8.DeepView Imaging

DeepView was used to create a 3D structure of OXA-66, OXA-69, OXA-71, OXA-64. They are shown below in figures 19, 20, 21, 22 respectively.

The DeepView software was used to calculate the distance in Å between the SERINE active site and the respective amino acid that are different than those of OXA-64. A distance of less than 10 Å was used to signify if the amino acid change had any significant effect on the active site and thus the bacteria, or not.

The positions of the amino acid, and properties of each amino acid, which are close to the 67 Serine active site in *Acinetobacter baumannii*, are described in table 8 below. Changes in the amino acids in each OXA type enzyme have not shown to have distances of less than 10 Å. Therefore it was concluded that the resistance or properties were unaltered by the change in amino acid types, but rather it could be something else.

Table 8. The positions and properties of each amino acid

Amino Acid Position	Amino Acid	Properties
37	Glutamic acid Valine Aspartic Acid	Polar, negative pH Nonpolar, neutral Polar, negative pH
39	Glycine Alanine	Nonpolar, neutral pH Nonpolar, neutral pH
97	Alanine Threonine	Nonpolar, neutral Polar, neutral
109	Glutamine Lysine Glutamic acid	Polar, neutral pH Nonpolar, neutral pH Polar, negative pH
196	Proline Glutamine	Nonpolar, neutral Polar, neutral pH

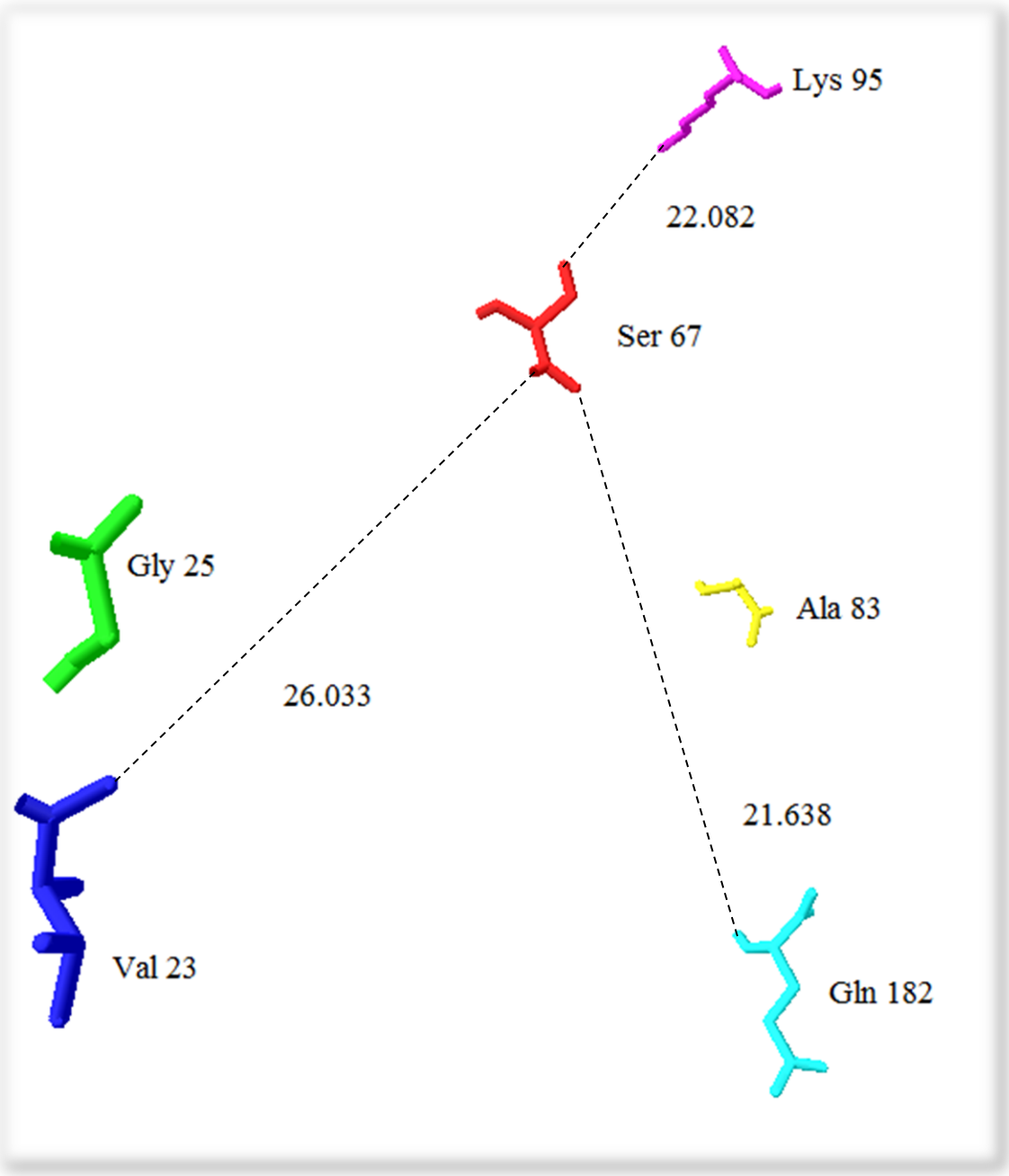


Figure 19. OXA-66 showing the distance between the Serine active site and: Valine (Val) = 26.033 Å, Glutamine (Gln) = 21.638 Å, and Lysine (Lys) = 22.082 Å.
The structure was created using DeepView.

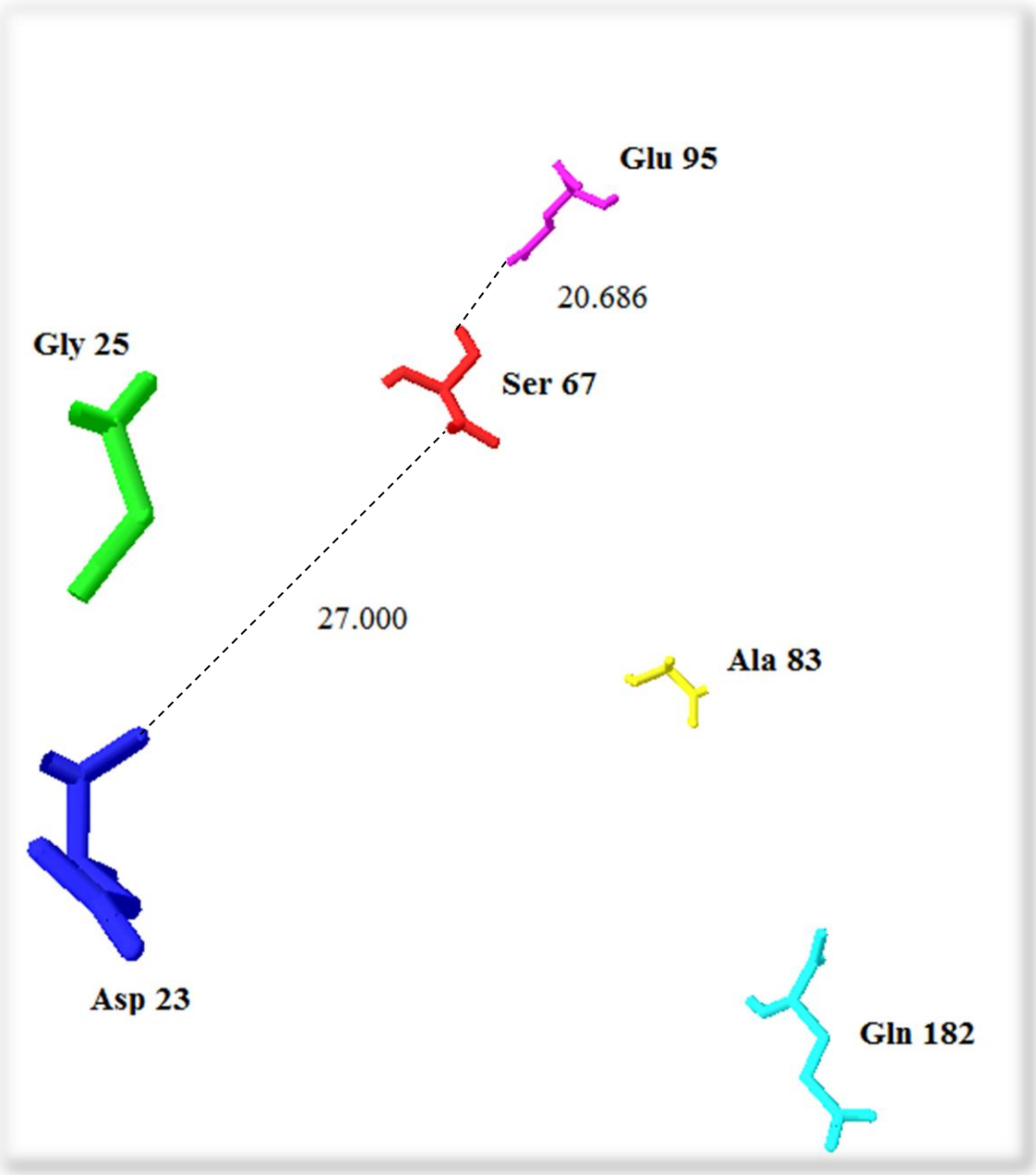


Figure 20. OXA-69 showing the distance between the Serine active site and: Aspartic acid (Asp) = 27.000 Å, and Glutamic acid (Glu) = 20.686 Å. The structure was created using DeepView.

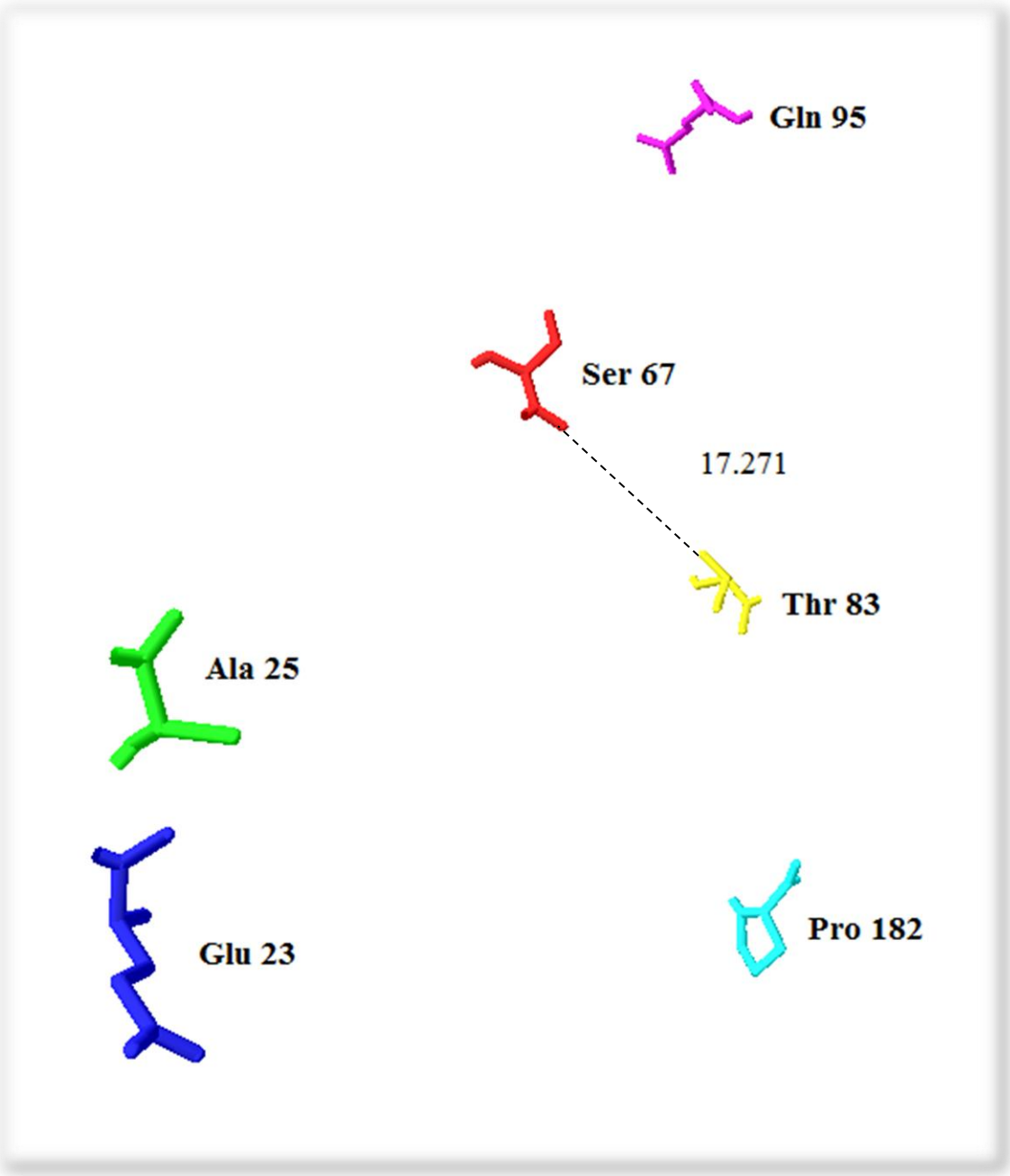


Figure 21. OXA-71 showing the distance between the Serine active site and: Threonine (Thr) = 17.271 Å. The structure was created using DeepView.

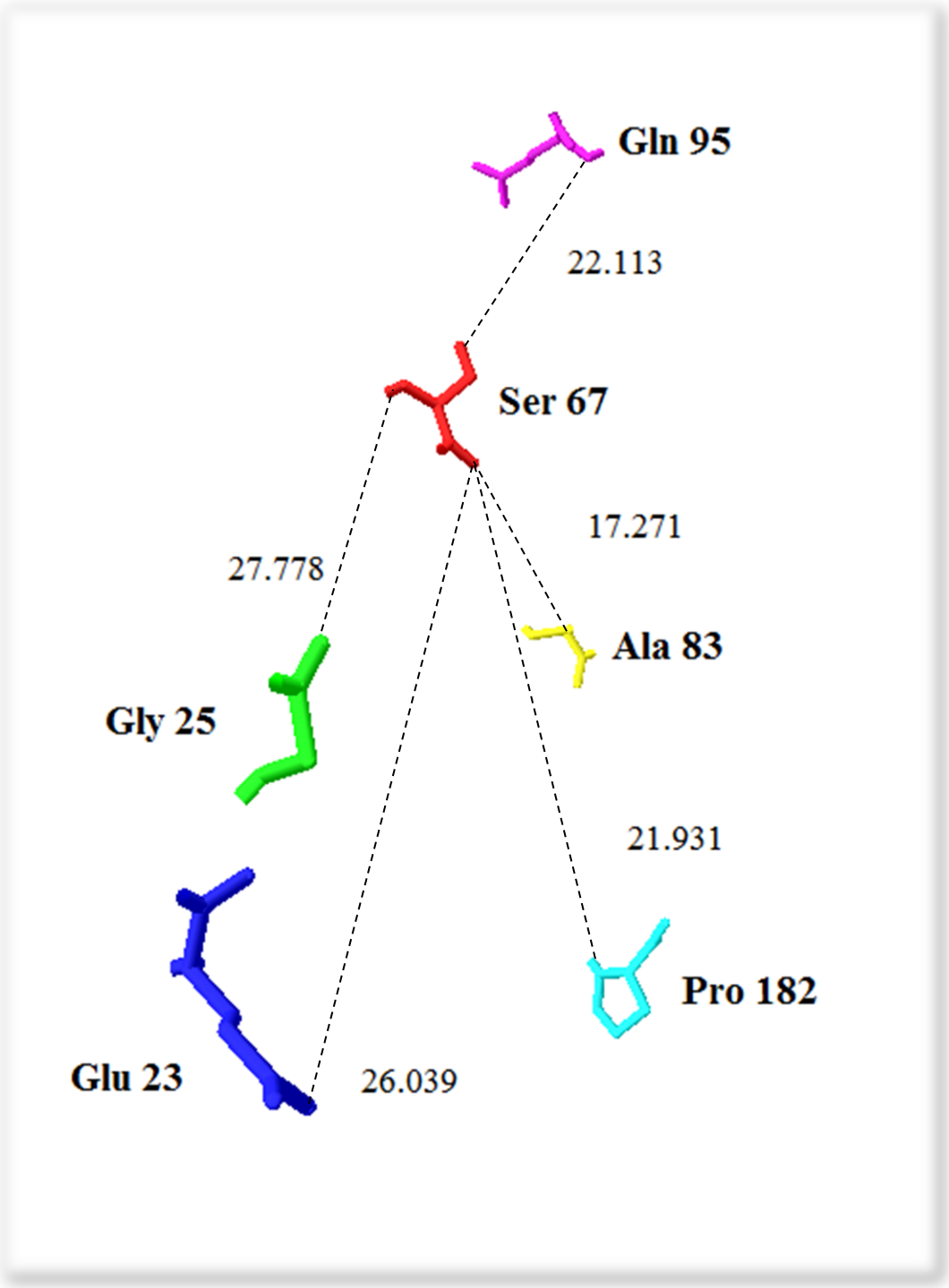


Figure 22. OXA-64 showing the distance between the Serine active site and: Glycine (Gly) = 27.778 Å, Glutamic acid (Glu) = 26.039 Å, Proline (Pro) = 21.931 Å, Alanine (Ala) = 17.271 Å, and Glutamine (Gln) = 22.113 Å. The structure was created using DeepView

3.9.Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-Field Gel electrophoresis (PFGE) is considered to be the standard method for genotyping a large variety of bacterial organisms, which has helped it become a major method in molecular diagnosis and epidemiology. In clinical microbiology, the detection and proper identification of virulent strains is very important, for only if and once a contagious strain is identified, the suitable medication may be given. The clonality of strains, whether they belong to the same clone group, or are spontaneous bacterial infections, can be identified using PFGE, which makes it a crucial epidemiological tool as well as a hospital diagnostic lab tool. Furthermore, the use of PFGE helps in the understanding of the phylogeny of each strain, providing information on horizontal gene transfer and gene conversion, genomic rearrangements, and the modular organization of chromosomes (Lahti, 1996).

Interpreting and understanding the epidemiology and clonal type of an organism may be ascertained by PFGE typing, and comparing the different bands of DNA from the genome of *A. baumannii* strains, following digestion with the restriction endonuclease enzyme *Apa*1 and analysing the generated fragments (Seifert *et al*., 1994a).

PFGE typing was used to analyse 88 clinical isolates of *Acinetobacter baumannii* obtained from the Mubarak Al-Kabeer hospital over the three year period 2006-2008. Their gels are shown in figures 25 to 31 below.

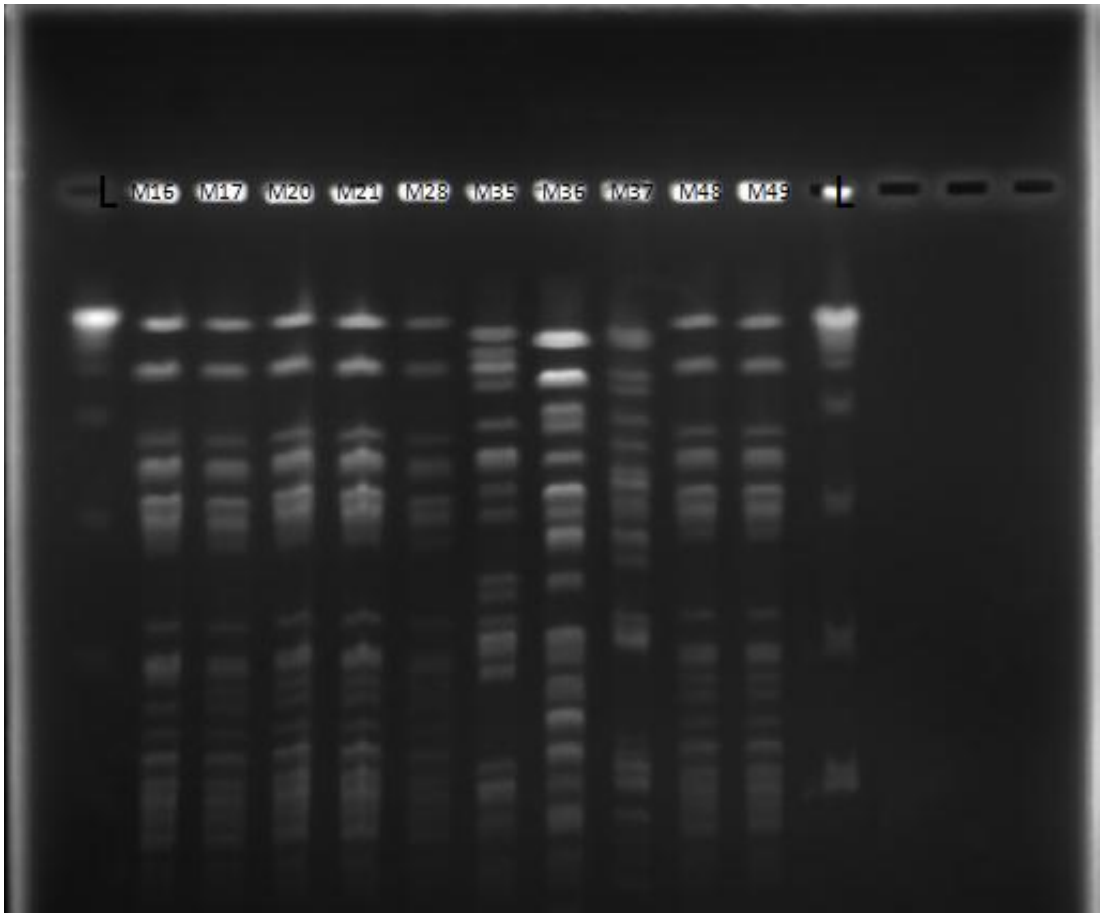


Figure 23. PFGE patterns showing isolates M16-M49.

Lane L: Lambda ladder PFG markers standard size. Lane 1: isolate M16. Lane 2: isolate M17. Lane 3: isolate M20. Lane 4: isolate M21. Lane 5: isolate M28. Lane 6: isolate M35. Lane 7: isolate M36. Lane 8:
isolate M37. Lane 9: isolate M48. Lane 10: isolate M49.

According to the sequencing results, and to better describe the Gel above, the respective isolates and their OXA-types are mentioned: Isolates M16,M17,M20,M21,M28,M48,M49 are positive for OXA-64. Isolates M35,M37 are positive for OXA-69. Isolates M36 was positive for OXA-117. Apa1 endonuclease was used for this gel.

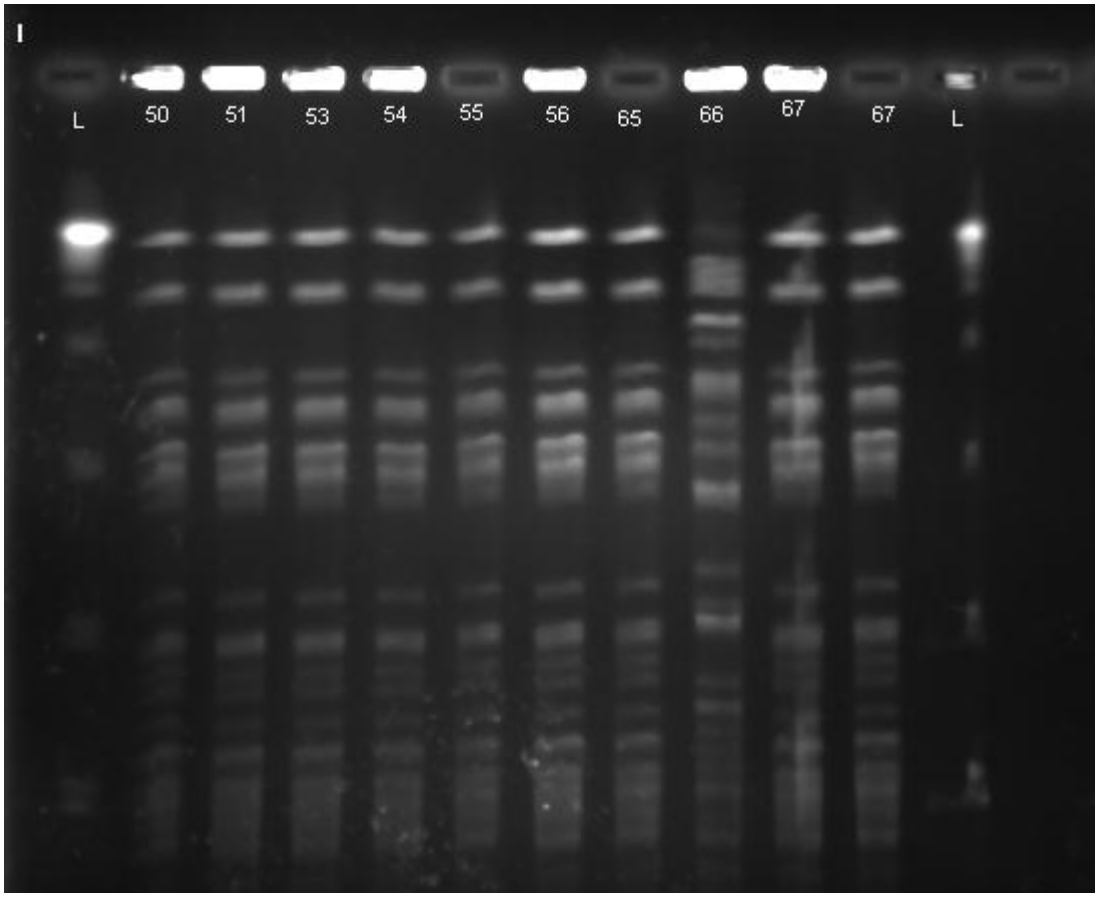


Figure 24. PFGE patterns of isolates M50-M67.

Lanes M: Lambda ladder PFG markers standard size. Lane 1: isolate M50. Lane 2: isolate M51. Lane 3: isolate M53. Lane 4: isolate M54.
Lane 5: isolate M55. Lane 6: isolate M56. Lane 7: isolate M66. Lane 8:
isolate M67. Lane 9: isolate M68.

According to the sequencing results, and to better describe the Gel above, the respective isolates and their OXA-types are mentioned: Isolates M50, M51, M53, M54, M55, M56, M65, M67 were all positive for OXA-64. Isolate M66 was positive for OXA-66. Apa1 endonuclease was used for this gel.

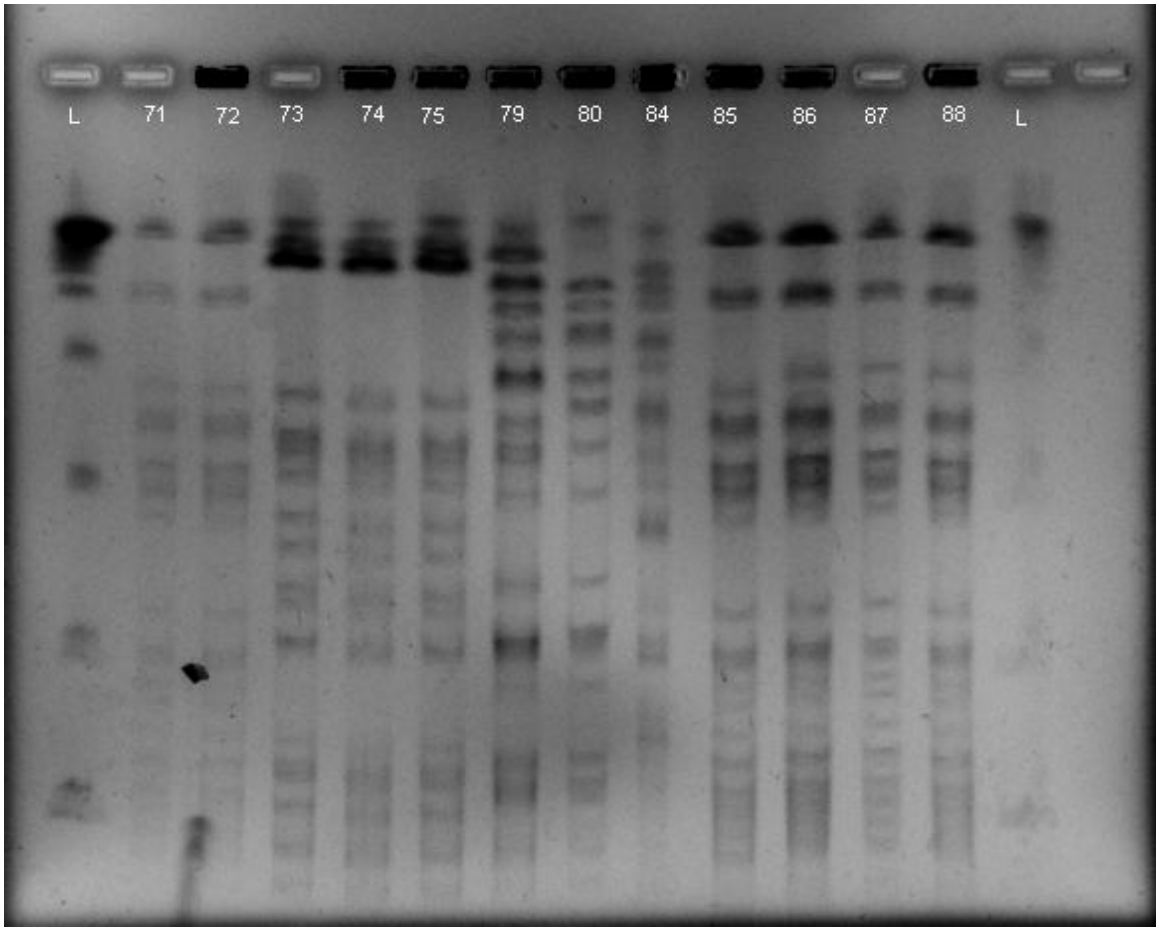


Figure 25. PFGE patterns of isolates M71-M88.

Lanes M: Lambda ladder PFG markers standard size. Lane 1: isolate M71. Lane 2: isolate M72. Lane 3: isolate M73. Lane 4: isolate M74.
Lane 5: isolate M75. Lane 6: isolate M79. Lane 7: isolate M80. Lane 8: isolate M84. Lane 9: isolate M85. Lane 10: isolate M86. Lane 9: isolate M87. Lane 11: isolate M88.

According to the sequencing results, and to better describe the Gel above, the respective isolates and their OXA-types are mentioned: Isolates M71, M72, M79, M80, M84, M85, M86, M87, M88 were all positive for OXA-64. Isolates M73, M74, M75 were positive for OXA-98. Isolate M84 was positive for OXA-66. Apa1 endonuclease was used for this gel.

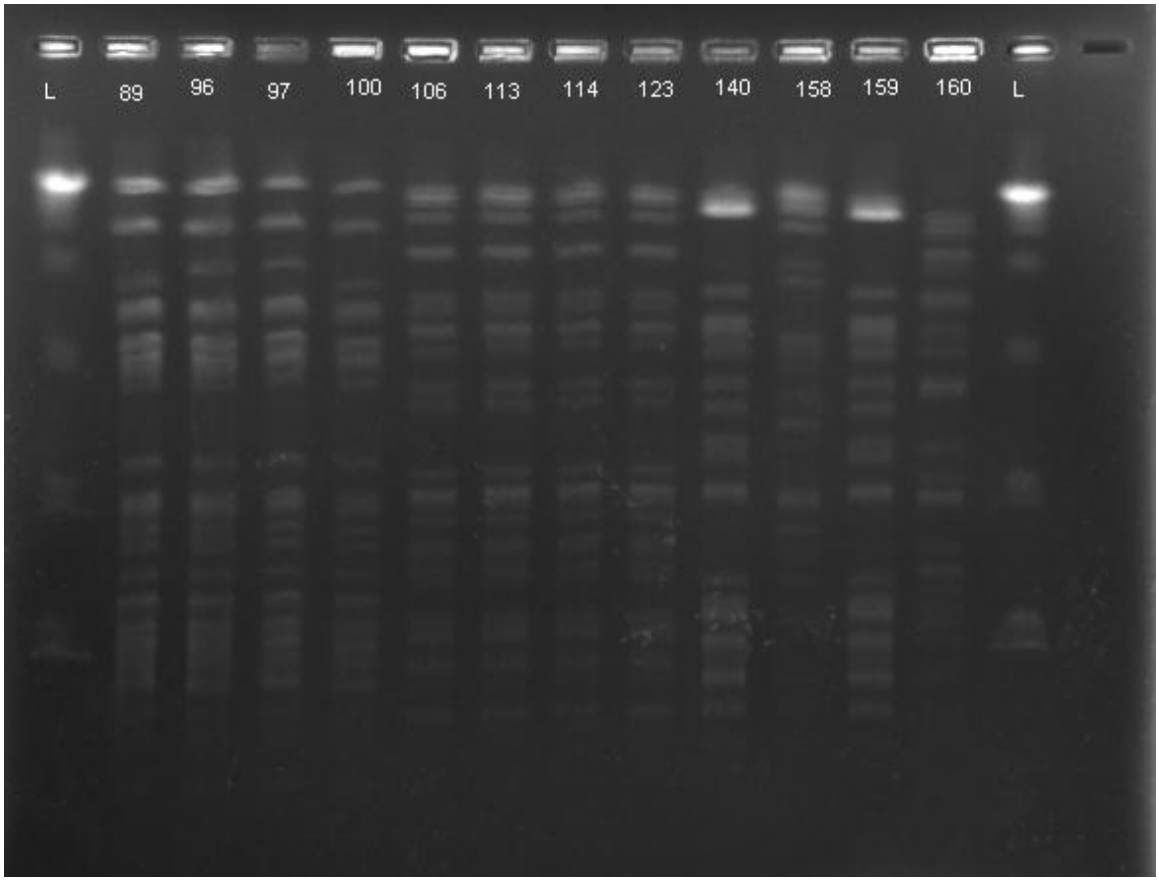


Figure 26. PFGE patterns of isolates M89-M160.

Lanes M: Lambda ladder PFG markers standard size. Lane 1: isolate M89. Lane 2: isolate M96. Lane 3: isolate M97. Lane 4: isolate M100. Lane 5: isolate M106. Lane 6: isolate M113. Lane 7: isolate M114. Lane 8: isolate M123. Lane 9: isolate M140. Lane 10: isolate M158. Lane 9: isolate M159. Lane 11: isolate M160.

According to the sequencing results, and to better describe the Gel above, the respective isolates and their OXA-types are mentioned: Isolates M89, M96, M97, M100, M123 were all positive for OXA-64. Isolates M106, M113, M114 were positive for OXA-69. Isolates M160 was positive for OXA-66. Isolates 158 and 159 are positive for OXA-71 and OXA-70 respectively. Apa1 endonuclease was used for this gel.

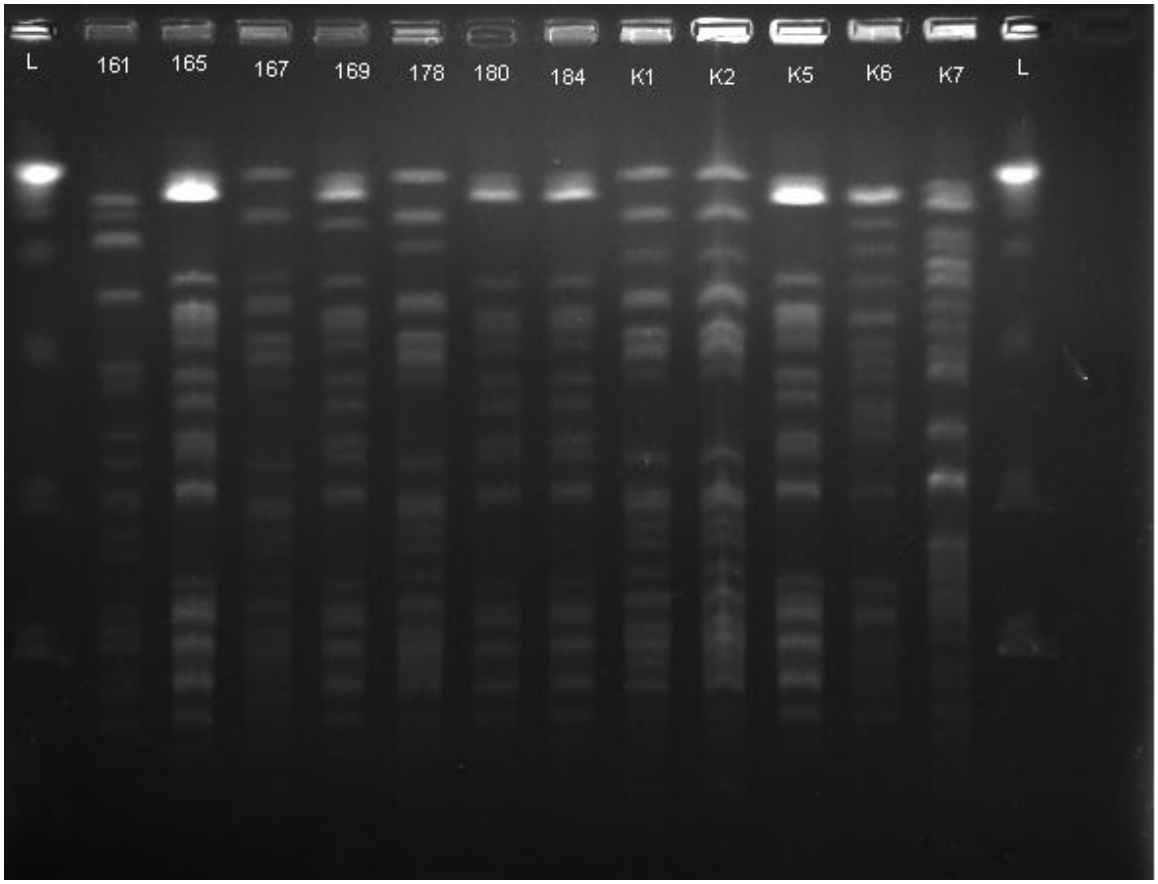


Figure 27. PFGE patterns of isolates M161-K7.

Lanes M: Lambda ladder PFG markers standard size. Lane 1: isolate M161. Lane 2: isolate M165. Lane 3: isolate M167. Lane 4: isolate M169. Lane 5: isolate M178. Lane 6: isolate M180. Lane 7: isolate M184. Lane 8: isolate K1. Lane 9: isolate K2. Lane 10: isolate K3. Lane 9: isolate K5. Lane 11: isolate K6. Lane 12: isolate K7

According to the sequencing results, and to better describe the Gel above, the respective isolates and their OXA-types are mentioned: Isolates M167, M178, K1, K2, K7 were all positive for OXA-64. Isolates M161, M165, M169, M180, M184, K5, K6 were all positive for OXA-98.

Apa1 endonuclease was used for this gel.

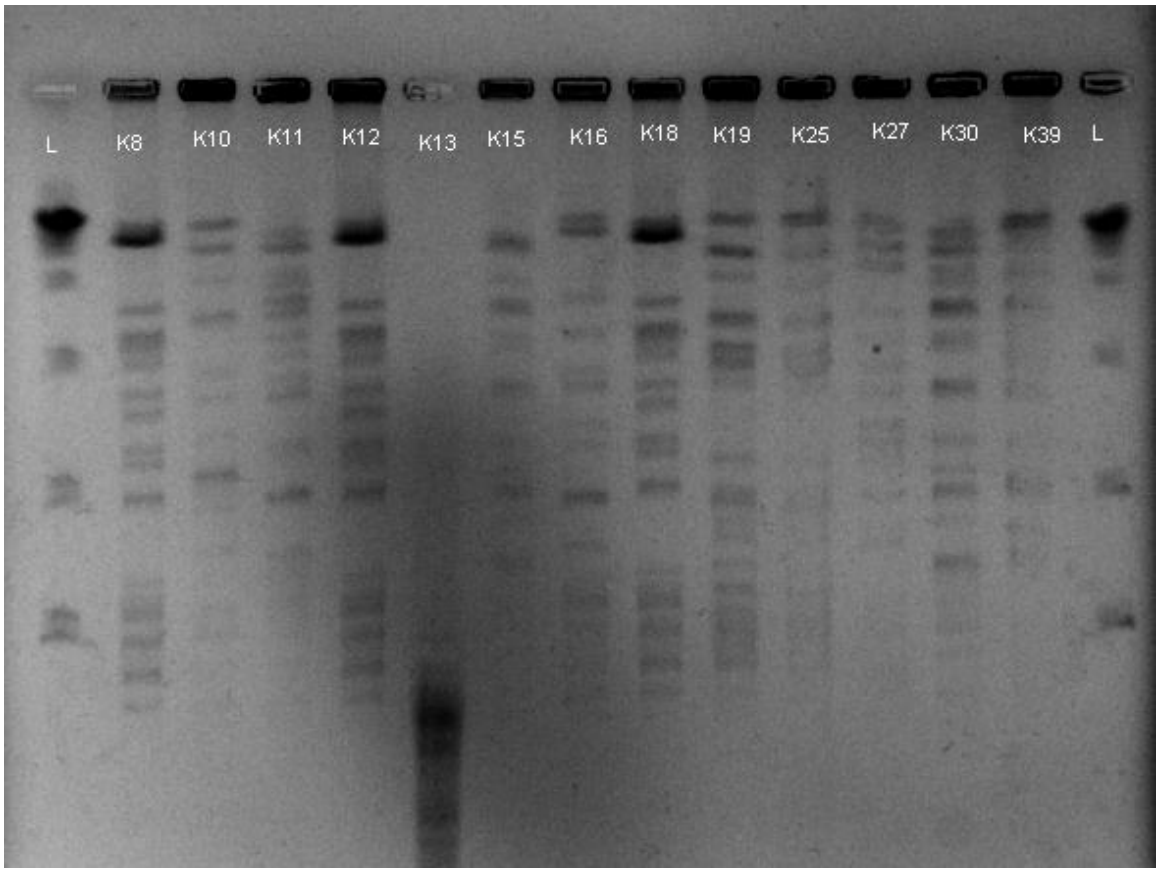


Figure 28. PFGE patterns of isolates K8-K39.

Lanes M: Lambda ladder PFG markers standard size. Lane 1: isolate K8. Lane 2: isolate K10. Lane 3: isolate K11. Lane 4: isolate K12. Lane 5: isolate K13. Lane 6: isolate K15. Lane 7: isolate K16. Lane 8: isolate K18. Lane 9: isolate K19. Lane 10: isolate K25. Lane 9: isolate K27. Lane 11: isolate K30. Lane 12: isolate K39

According to the sequencing results, and to better describe the Gel above, the respective isolates and their OXA-types are mentioned: Isolates K16, K25 were positive for OXA-64. Isolates K11, K15, K30, K39 were positive for OXA-66. Isolates K10 and K27 were positive for OXA-71. Isolates K8, K12, K18, K19 were positive for OXA-98. Apa1 endonuclease was used for this gel.

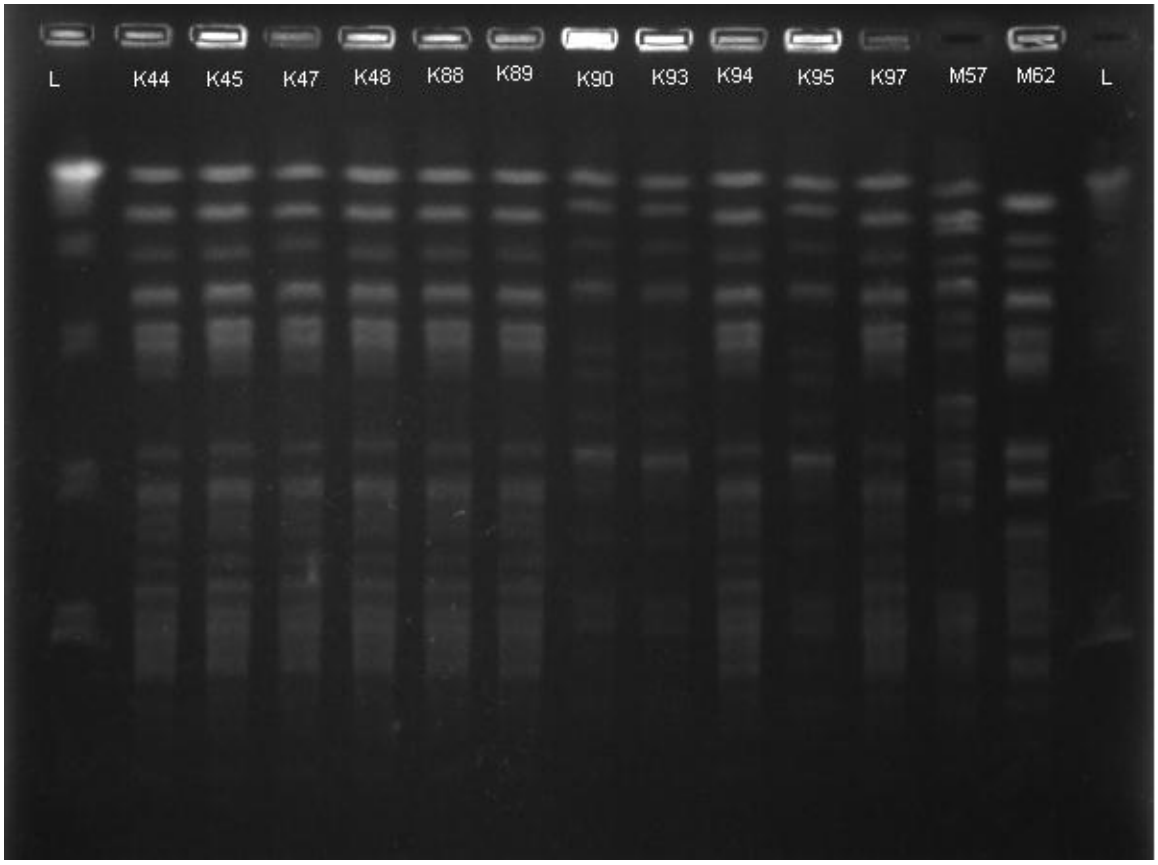


Figure 29. PFGE patterns of isolates K44-M62.

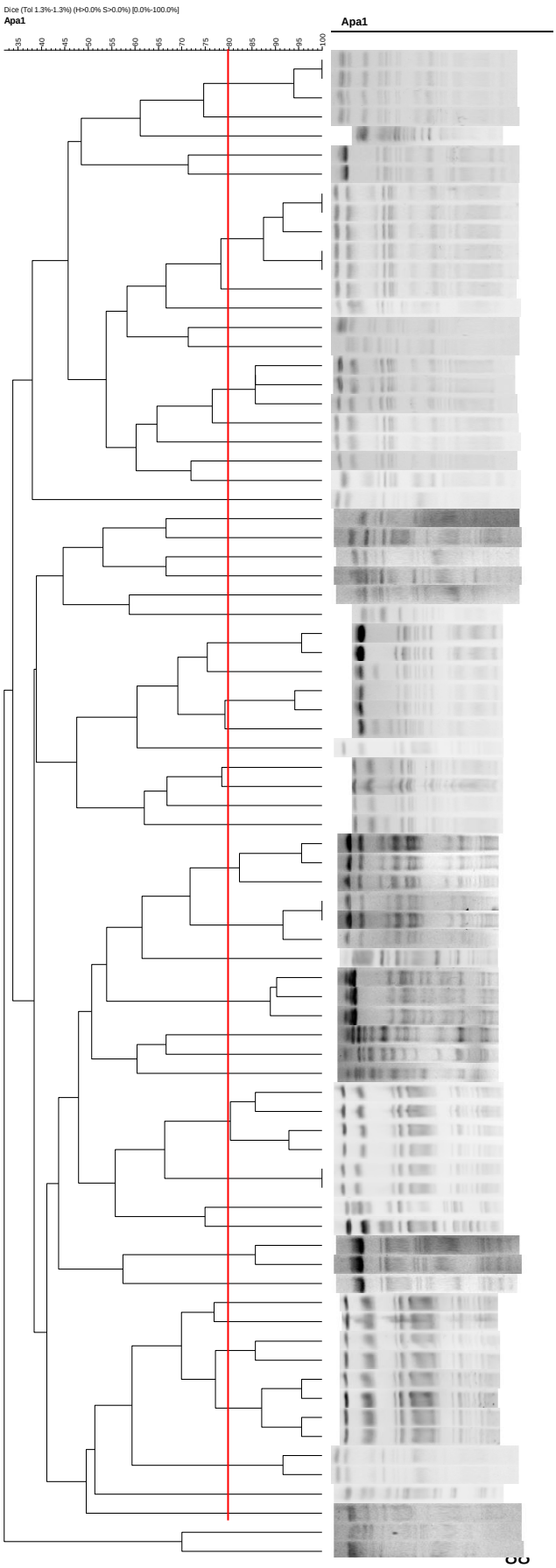
Lanes M: Lambda ladder PFG markers standard size. Lane 1: isolate K44. Lane 2: isolate K45. Lane 3: isolate K47. Lane 4: isolate K48 Lane 5: isolate K88. Lane 6: isolate K89. Lane 7: isolate K90. Lane 8: isolate K93. Lane 9: isolate K94. Lane 10: isolate K95. Lane 9: isolate K97. Lane 11: isolate M57. Lane 12: isolate M62.

According to the sequencing results, and to better describe the Gel above, the respective isolates and their OXA-types are mentioned: Isolates K44, K45, K47, K48, K89, K94, K97 were positive for OXA-64. Isolates K90, K93, K95 were positive to OXA-71. Isolates M57 and M62 were positive to OXA-51 and OXA-98 respectively. Apa1 endonuclease was used for this gel.

A Dendogram, shown in figure 30, was made to help better understand the clonality of the isolates, and their relatedness to each other. We could see that the OXA-64 isolates were all clonal, from the years 2006 till 2008. The same is true about OXA-98, OXA-66, and OXA-71 which has patterns that are the same throughout the 3 years of study. They are all more than 80% similar and in some cases over 90%. There were no clear relatedness to the other OXAs in this study, which mean that they are not clones, and individual cases.

This shows that the 4 major clones OXA-64, OXA-71, OXA98, and OXA-66 have survived any attempt by the Mubarak Al-Kabeer hospital to eradicate the pathogen from their patients. One could also deduce that, if these patients were not infected nosocomially, then these four OXA-types are more wide spread in Kuwait than expected, which warrants further research into the depth of spread and dissemination of these four OXA types in Kuwait.

Figure 30. Below shows a Dendogram showing the Clonality of the Isolates, along with the year of collection, gender of the patients collected from, MIC to Imipenem and Meropenem, and OXA-types of the *Acinetobacter baumannii* samples. The red vertical line denotes 80% relatedness.



M106	A. baumannii	Kuwait	Endotracheal	2007	F	4	2	OXA-69
M113	A. baumannii	Kuwait	Endotracheal	2007	F	4	2	OXA-69
M114	A. baumannii	Kuwait	Endotracheal	2007	F	1	0.25	OXA-69
M123	A. baumannii	Kuwait	Urine	2007	M	0.5	0.12	OXA-64
K7	A. baumannii	Kuwait	Blood	2008	F	8	8	OXA-66
M140	A. baumannii	Kuwait	Pus Swab	2007	F	0.5	0.06	OXA-98
M159	A. baumannii	Kuwait	Endotracheal	2007	F	8	8	OXA-70
K47	A. baumannii	Kuwait	Blood	2008	F	2	4	OXA-64
K48	A. baumannii	Kuwait	Endotracheal	2008	F	8	8	OXA-64
K45	A. baumannii	Kuwait	Urine	2008	F	8	2	OXA-64
K88	A. baumannii	Kuwait	Blood	2008	F	8	2	OXA-64
K89	A. baumannii	Kuwait	Blood	2008	F	16	16	OXA-64
K44	A. baumannii	Kuwait	Endotracheal	2008	M	8	2	OXA-64
M57	A. baumannii	Kuwait	ICU	2006	F	0.5	0.12	OXA-51
M158	A. baumannii	Kuwait	Endotracheal	2007	M	2	1	OXA-71
M160	A. baumannii	Kuwait	Sputum	2007	M	4	1	OXA-66
M89	A. baumannii	Kuwait	Skin Swab	2007	M	8	8	OXA-64
M96	A. baumannii	Kuwait	Blood	2007	M	16	16	OXA-64
M97	A. baumannii	Kuwait	Blood	2007	M	16	8	OXA-64
K94	A. baumannii	Kuwait	Tip	2008	F	16	16	OXA-64
K97	A. baumannii	Kuwait	Blood	2008	F	32	16	OXA-64
M100	A. baumannii	Kuwait	Blood	2007	F	8	4	OXA-64
M62	A. baumannii	Kuwait	Blood	2006	F	4	4	OXA-98
K95	A. baumannii	Kuwait	Aspiration Fluid	2008	F	0.25	0.03	OXA-71
K15	A. baumannii	Kuwait	Endotracheal	2008	F	16	16	OXA-66
K19	A. baumannii	Kuwait	Urine	2008	M	16	8	OXA-98
K10	A. baumannii	Kuwait	Blood	2008	M	8	4	OXA-71
K30	A. baumannii	Kuwait	Tip	2008	F	0.06	0.015	OXA-66
K11	A. baumannii	Kuwait	Blood	2008	F	16	8	OXA-66
M161	A. baumannii	Kuwait	Blood	2007	M	32	16	OXA-98
K5	A. baumannii	Kuwait	Blood	2008	M	16	4	OXA-98
M165	A. baumannii	Kuwait	Blood	2007	M	16	16	OXA-98
M169	A. baumannii	Kuwait	Exit site Swab	2007	M	8	8	OXA-98
M180	A. baumannii	Kuwait	Pus Swab	2007	F	16	16	OXA-98
M184	A. baumannii	Kuwait	Pus Swab	2007	F	16	16	OXA-98
K6	A. baumannii	Kuwait	Blood	2008	F	4	8	OXA-98
M28	A. baumannii	Kuwait	Urine	2006	M	2	4	OXA-64
K1	A. baumannii	Kuwait	Blood	2008	M	16	16	OXA-64
K2	A. baumannii	Kuwait	Endotracheal	2008	M	0.5	0.25	OXA-64
M167	A. baumannii	Kuwait	Aspiration Fluid	2007	M	0.5	0.5	OXA-64
M178	A. baumannii	Kuwait	Pus Swab	2007	M	8	16	OXA-64
M86	A. baumannii	Kuwait	Blood	2007	M	4	2	OXA-64
M88	A. baumannii	Kuwait	Blood	2007	M	16	8	OXA-64
M87	A. baumannii	Kuwait	Blood	2007	M	16	16	OXA-64
M72	A. baumannii	Kuwait	Tip	2006	F	0.5	0.25	OXA-64
M85	A. baumannii	Kuwait	Blood	2007	M	8	8	OXA-64
M71	A. baumannii	Kuwait	Tip	2006	F	0.25	0.25	OXA-64
M66	A. baumannii	Kuwait	Endotracheal	2006	M	16	8	OXA-66
M73	A. baumannii	Kuwait	Pus Swab	2006	M	0.5	0.12	OXA-98
M74	A. baumannii	Kuwait	Tissue	2006	M	1	0.25	OXA-98
M75	A. baumannii	Kuwait	Tissue	2007	M	0.25	0.25	OXA-98
M79	A. baumannii	Kuwait	Urine	2007	F	0.25	0.03	OXA-64
M80	A. baumannii	Kuwait	Exit site Swab	2007	F	0.12	0.06	OXA-64
M84	A. baumannii	Kuwait	Tip	2007	F	0.5	0.5	OXA-66
M20	A. baumannii	Kuwait	Rectal Swab	2006	M	1	1	OXA-64
M21	A. baumannii	Kuwait	Endotracheal	2006	F	0.25	0.25	OXA-64
M16	A. baumannii	Kuwait	Rectal Swab	2006	M	0.5	0.25	OXA-64
M17	A. baumannii	Kuwait	ICU	2006	M	1	4	OXA-64
M48	A. baumannii	Kuwait	Blood	2006	F	16	16	OXA-64
M49	A. baumannii	Kuwait	Blood	2006	M	2	2	OXA-64
M35	A. baumannii	Kuwait	Tip	2006	M	0.5	0.25	OXA-69
M36	A. baumannii	Kuwait	Urine	2006	M	16	16	OXA-117
K12	A. baumannii	Kuwait	Tip	2008	M	4	2	OXA-98
K18	A. baumannii	Kuwait	Blood	2008	M	32	16	OXA-66
K8	A. baumannii	Kuwait	Urine	2008	M	16	16	OXA-98
M65	A. baumannii	Kuwait	Blood	2006	M	8	4	OXA-64
M67	A. baumannii	Kuwait	Blood	2006	F	16	16	OXA-64
M50	A. baumannii	Kuwait	Blood	2006	M	2	1	OXA-64
M51	A. baumannii	Kuwait	Blood	2006	M	2	0.5	OXA-64
M55	A. baumannii	Kuwait	Blood	2006	M	16	8	OXA-64
M56	A. baumannii	Kuwait	Urine	2006	M	2	0.5	OXA-64
M53	A. baumannii	Kuwait	Blood	2006	M	16	8	OXA-64
M54	A. baumannii	Kuwait	Blood	2006	M	8	8	OXA-64
K90	A. baumannii	Kuwait	Blood	2008	F	4	4	OXA-71
K93	A. baumannii	Kuwait	Endotracheal	2008	F	16	16	OXA-71
M37	A. baumannii	Kuwait	Urine	2006	F	2	2	OXA-69
K25	A. baumannii	Kuwait	Endotracheal	2008	M	0.5	0.5	OXA-64
K27	A. baumannii	Kuwait	Pus Swab	2008	M	4	4	OXA-71
K39	A. baumannii	Kuwait	Tip	2008	M	0.12	0.03	OXA-66

4. Discussion

Bacteria of the genus *Acinetobacter* are important opportunistic pathogens that are responsible for nosocomial infections. *Acinetobacter* species are highly resistant to commonly used antibiotics such as penicillins, cephalosporins, aminoglycosides and fluoroquinolones by intrinsic and acquired mechanisms. They are also gradually becoming resistant to carbapenems, as this study has shown, in Kuwait.

Hospitals have long served as reservoirs for the transmission of pathogenic bacteria, and this has become a problem in Kuwait. Regrettably, little research has been devoted to investigating the prevalence, resistance and pathogenicity of *Acinetobacter baumannii* in Kuwait Hospitals.
This study examined the resistance profiles and identified the genotypic changes in *Acinetobacter baumannii* as it spread through Mubarak Al-Kabeer Hospital during the years 2006-2008.

Research in Kuwait has shown that there have been numerous outbreaks of *Acinetobacter baumannii* in several hospitals (Al-Sweih *et al*., 2011). A variation in the number of clone types was also shown in the study. This coincides with the results that I have shown, where in the Mubarak Al-Kabeer Hospital, one of the major hospitals in Kuwait, the variation of *Acinetobacter baumannii* clones ranged from European Clone 1, 2, and 3.

This study demonstrated high resistance to carbapenems in *Acinetobacter baumannii* isolates that were collected from patients in Mubarak Al-Kabeer Hospital in Kuwait. The increase in

Acinetobacter baumannii carbapenem resistance is becoming a worldwide phenomenon, but with some variation in the carbapenem resistance numbers from one geographical area to another. There have also been reports of an increase of 6% in 1998 which increased to approximately 29% in 2005 of Meropenem resistant *Acinetobacter baumannii* (Perez *et al*,. 2007). This study also confirms this report since there was a noticeable gradual increase in the number of Imipenem and Meropenem resistant *Acinetobacter baumannii* isolates over the course of this study.

The presence of *bla*-OXA-51-like beta-lactamases in *Acinetobacter baumannii* has a major role in the resistance to carbapenems. These beta-lactamases which have a role in carbapenem resistance appear to be globally spread (Poirel and Nordmann, 2006a), and recent studies also included Middle Eastern countries such as Iraq (Scott *et al*., 2007), Kuwait (Al-Sweih *et al*., 2011), Saudi Arabia (AlSultan *et al*., 2009), Bahrain (Mugnier *et al*., 2009) and the United Arab Emirates (Mugnier et al., 2008).

The identification of *Acinetobacter baumannii* phenotypically is a difficult problem due to significant phenotypic overlapping between strains, which are genotypically closely related to each other. (Dolzani *et al*., 1995)

The *bla*OXA-51-like gene is present intrinsically and is naturally located in all *Acinetobacter baumannii* (Brown *et al*., 2006), therefore the OXA-69 primer was used to identify the isolates in

the PCR identification part, and also give a complete gene sequence later in the sequencing part of the study.

From the 120 samples, 110 were identified as *Acinetobacter baumannii* positive by the VITEK-2 system method, which is a phenotypic method of identification.

The percentage of female patients with positive *Acinetobacter baumannii* was calculated as being 49.1%, and the percentage of male patients with positive *Acinetobacter baumannii* was 50.9%.

The majority of samples that have been identified phenotypically via the VITEK-2 system as positive for *Acinetobacter baumannii* were collected from: blood, which totalled 42 samples, or 38.09% of the total positives, there may be several reasons for this, however in the absence of clinical records of the patients, it is difficult to reach a clear conclusion explaining it. Further study is needed in the hospital ward and the individual patients to better understand the nature of their disease and cause of it. One could speculate that open wound infections could provide a direct opening to the blood and internal tissues that could help the pathogen in infecting the patients.

The other sample sources included urine, which totaled 16, or 16.5% of the total positive, endotracheal samples, which totaled 14, or 12.7% of the total positive sample, and the fourth prevalent source was from Tip samples, that totaled 10, or 9.1%. According to the genotypic test of PCR, Of the 110 samples, only 88 were identified as positive for *Acinetobacter baumannii,* by

OXA-69 PCR. This difference in results suggests that the VITEK-2 system in this study had an error of 20% and thus is probably unreliable as a method to detect *Acinetobacter baumannii*

While the VITEK-2 system is a faster, less expensive and less laborious method, one should not ignore the possible erroneous or inaccurate identification it may provide, and in some cases, using genotypic methods, such as PCR for OXA-69, for identification are a more solid source of identifying *Acinetobacter baumannii*.

From the genotypic method, we also deduce that the percentage of males and females positive for *Acinetobacter baumannii* was calculated as being 56.8% for females and 43.2% for males, not denoting any major relationship between the sex of the patients and their chances of being infected with *Acinetobacter baumannii*.

The MIC tests showed a noticeable increase in the number of resistant bacteria to Imipenem and Meropenem over the year 2006 to 2008. This is concordant to other results in Kuwait (Al-Sweih *et al*., 2011) and solidifies what is already becoming a regional (Scott *et al*., 2007) and global event (Perez *et al*., 2007).

The MIC study also showed that the highest percentage of Imipenem and Meropenem resistant samples was found in 2008. Inversely, the lowest percentage of resistant samples was found in 2006.

We can clearly see a gradual increase in the overall percentage of carbapenem resistant samples, signifying a gradual increase in the overall resistance of *Acinetobacter baumannii* to carbapenems, in Kuwait. This slow and gradual resistance evolution could be due to several factors including the over-prescription of antibiotics by GPs in Kuwait, which are helping the bacteria to slowly evolving resistance to carbapenems, and becoming harder to treat.

Acinetobacter baumannii by nature is a soil inhabiting, ubiquitous bacteria. The rise in the number of outbreaks in the surrounding middle-east countries has led me to believe that dust winds could also have a role to play in carrying and spreading the bacterium from one place to another. A study showed the presence of *Acinetobacter baumannii* in soil samples from Iraq and Kuwait (Scott *et al*., 2007). Further environmental research needs to be conducted to see if this hypothesis is true.

Another factor that should not be ignored is that the expatriates in Kuwait form 2/3 of the total population in Kuwait. Their regular travel back and forth between Asian countries such as India and Pakistan, and North African countries such as Egypt, could also be considered as potential risk factors as they may be carriers of this bacterium.

The analysis of the phylogeny of *Acinetobacter baumannii* as a species along with the *bla*OXA-51-like genes provided considerable evidence that within the species there are discrete lineages, and these lineages have been evolving independently of one another for a significant time period. The *bla*OXA-51-like genes, which are associated with these lineages, have also been co-evolving (Evans *et al*., 2007).

The total number of OXA samples that were sequenced was 88, with OXA-64 being the major OXA-type, and was found in the 44 samples. OXA-98 was found to be the second most prevalent, and was found in 16 samples; OXA-66 was third most prevalent, and found in 9 samples, and OXA-71 was found in 6 samples.

This study was the first to show the OXA-type distribution for *Acinetobacter baumannii* isolates from Kuwait. The study was also the first to identify a total of 10 OXA-types to be isolated from the Mubarak Al-Kabeer hospital in Kuwait, which is one of the Major hospitals in Kuwait.

To better understand the locations and relationships of each OXA-type in this study, an OXA-51-like enzyme map taken from Evans *et al*. (2007) and with the OXA-types discovered in this study are highlighted and shown in figure 33.

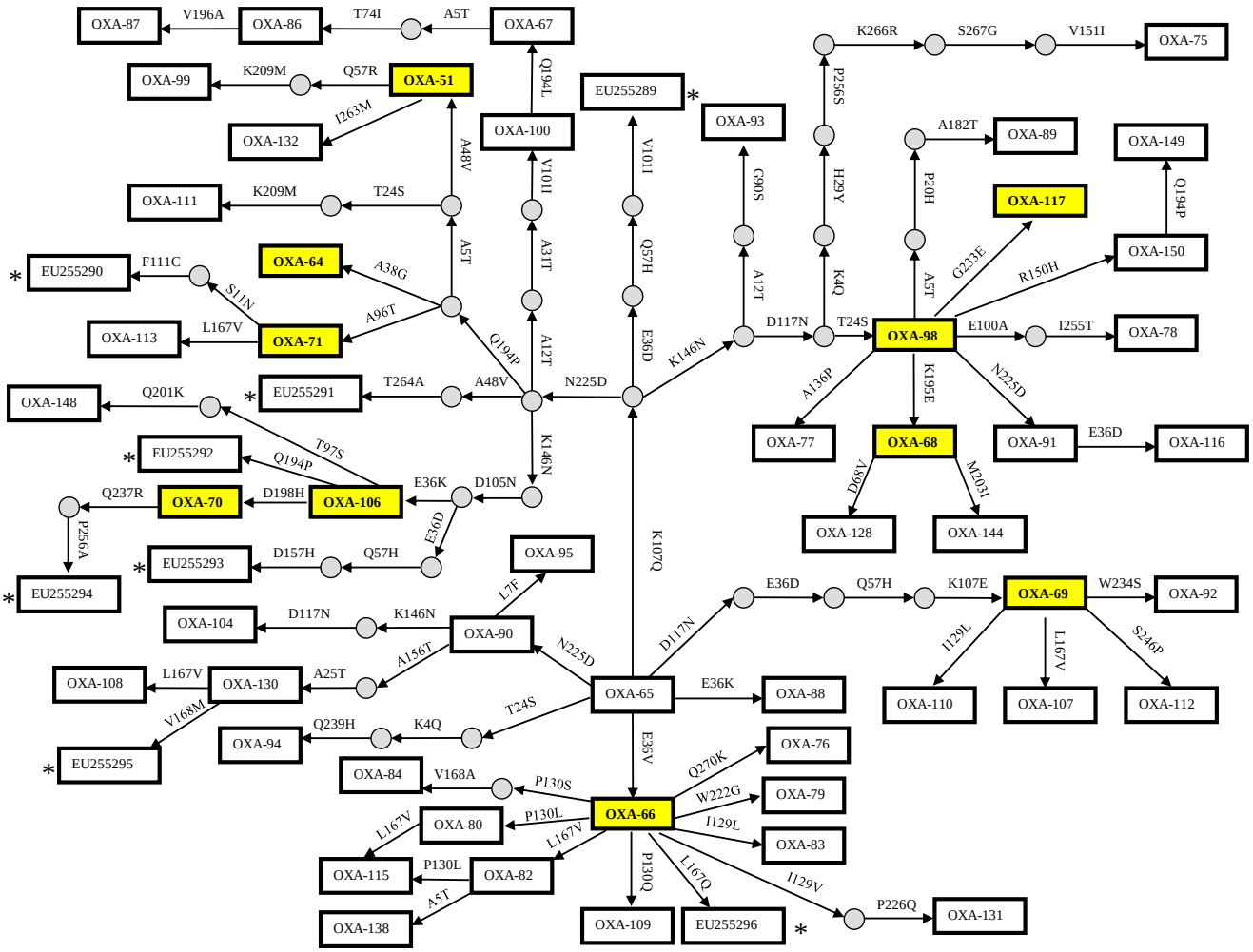


Figure 31. Showing the OXA-51-like enzyme map, and the highlighted OXAs are the ones found in this study.

According to the recent literature, OXA-64 in *Acinetobacter baumannii* samples has been worked on from samples from two nations so far, namely in Germany (Pfeifer *et al*., 2011), and samples from the United Arab Emirates, with the work being done at the University of Edinburgh by one of our fellow group members (Opazo *et al*., 2012). The complete gene sequence of OXA-64 gene was submitted and accepted by GenBank in 2005 by S. Brown and S.G.B. Amyes from the University of Edinburgh, under accession number: AY750907.1

One could speculate that one of the reasons why OXA-64 was found so readily through our study is because the clone was able to survive and flourish under the current circumstances at the hospital, and one could also speculate that if the patients were cross infected during outbreaks of that specific strain of *Acinetobacter baumannii* during their stay at the hospital.

The complete gene sequence of the novel enzyme OXA-117 which is located close to the $bla_{\text{OXA-98-like}}$ gene was discovered in this study, and the complete gene sequence was submitted to GenBank under the accession number: GQ423625.1.

Using DeepView software, changes in the amino acids in each OXA type enzyme have not shown to have distances of less than 10Å. It was therefore concluded that the resistance or properties of the *Acinetobacter baumannii* were unaltered by the change in amino acid types, but rather it could be something else. We can speculate that the resistance in the isolates may be due to non-enzymatic mechanisms such as porin defects or efflux pumps.

The PFGE results were put in a Dendogram to better understand the clonal relationship between each strain. We could see that almost all the OXA-64 isolates were clones, with a clonality of more than 80%, from the years 2006 till 2008. The same is true about OXA-98, OXA-66, and OXA-71 which has patterns that are the same throughout the 3 years of study. There are more than 80% similar and in some cases over 90%. There were no clear relatedness to the other OXA-types in this study; this could mean that they are not clones and that they are individual cases rather than outbreaks of a certain clone.

The 4 major clones OXA-64, OXA-71, OXA98, and OXA-66 have been able to survive and cross infected patients in outbreaks in the Mubarak Al-Kabeer hospital in Kuwait. One could also speculate that the details of the infection, where and when it occurred to the patient, could play a pivotal role in understanding if these four OXA-types are more wide spread in Kuwait, rather than just being enclosed and specific to the Mubarak Al-Kabeer hospital. Further research into the depth of spread and dissemination of these four OXA types in Kuwait could help us answer that question.

5. Conclusion

We can conclude from this study VITEK-2 should not be used to identify *Acinetobacter baumannii*. Furthermore, four major clones were found in the Mubarak Al-Kabeer hospital during the study period, three of the clones were closely associated with those found in Europe and elsewhere in the world, and one new clone, OXA-117, containing a $bla_{\text{OXA-98-like}}$ gene that appears to be more prevalent in this part of Asia. This was the first study that discovered and characterized OXA-types in Kuwait. OXA-64 was found to be the predominant OXA-type found in this study. The gradual increase in resistance to carbapenems over the study period warrants further attention and study of this resilient bacterium, and its ever evolving resistance to carbapenems.

6. References

1. AFZAL-SHAH, M., WOODFORD, N. & LIVERMORE, D. M. (2001). Characterization of OXA-25, OXA-26, and OXA-27, molecular class D β-lactamases associated with carbapenem resistance in clinical isolates of *Acinetobacter baumannii*. *Antimicrobial Agents Chemotherapy*, 45, 583-8.

2. ANSTEY, N. M., CURRIE, B.J., HASSELL, M., PALMER, D., DWYER, B. & SEIFERT, H. (2002). Community-acquired bacteremic *Acinetobacter* pneumonia in tropical Australia is caused by diverse strains of *Acinetobacter baumannii*, with carriage in the throat in at-risk groups. *Journal of Clinical Microbiology*, 40:685–686.

3. ALSULTAN, A.A., HAMOUDA, A., EVANS, B.A. & AMYES, S.G. (2009) *Acinetobacter baumannii*: emergence of four strains with novel bla(OXA-51-like) genes in patients with diabetes mellitus. *Journal of Chemotherapy*, 21(3):290-5.

4. AL-SWEIH, N.A., AL-HUBAIL, M.A. & ROTIMI V.O. (2009). Emergence of tigecycline and colistin resistance in Acinetobacter species isolated from patients in Kuwait hospitals. *Journal of Chemotherapy*, 23(1):13-6.

5. AL-SWEIH, NA., AL-HUBAIL, M. & ROTIMI, V.O. (2011). Three distinct clones of carbapenem-resistant *Acinetobacter baumannii* with high diversity of carbapenemases isolated from patients in two hospitals in Kuwait. *Journal of infection and public health*, 5(1):102-8.

6. BAUMANN, P. (1968). Isolation of *Acinetobacter* from soil and water. *J. Bacteriology*, 96:39–42.

7. BAUMANN, P., DOUDOROFF, M. & STANIER, R.Y. (1968). A study of the *Moraxella* group. II. Oxidative-negative species (genus *Acinetobacter*). *J. Bacteriology*, 95:1520–1541.

8. BERGOGNE-BEREZIN, E. & TOWNER, K. J. (1996). *Acinetobacter spp*. As nosocomial pathogens: *microbiological, clinical, and epidemiological features,* 9, 148-165.

9. BERLAU, J., AUCKEN, H., MALNICK, H. & PITT, T. (1999). Distribution of *Acinetobacter* species on skin of healthy humans. *Eur J Clin Microbiol Infect Dis,* 18, 179-183.

10. BERNARDS, A. T.,DIJKSHOORN, L.,VAN DER TOORN, J., BOCHNER, B.R. & VAN BOVEN, C.P. (1995). Phenotypic characterisation of *Acinetobacter* strains of 13 DNA-DNA hybridisation groups by means of the biolog system. *J. Med. Microbiol,* 42:113–119.

11. BERNARDS, A. T., VAN DER TOORN, J., VAN BOVEN, C.P. & DIJKSHOORN, L. (1996). Evaluation of the ability of a commercial system to identify *Acinetobacter* genomic species. *Eur. J. Clin. Microbiol. Infect. Dis,* 15:303–308.

12. BIRNBAUM, J., KAHAN, F. M., KROPP, H. & MACDONALD, J. S. (1985). Carbapenems, a new class of β-lactam antibiotics. Discovery and development of imipenem/cilastatin. *Am J Med,* 78, 3-21.

13. BOO, T. W., WALSH, F. & CROWLEY, B. (2009). Molecular characterization of carbapenem-resistant *Acinetobacter* species in an Irish university hospital: predominance of *Acinetobacter* genomic species 3. *J Med Microbiol*, 58, 209-216

14. BOU, G. & MARTINEZ-BELTRAN, J. (2000). Cloning, nucleotide sequencing and analysis of the gene encoding an AmpC β-lactamase in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*, 44, 428-32.

15. BOU, G., CERVERO, G., DOMINGUEZ, M. A., QUEREDA, C. & MARTINEZBELTRAN, J. (2000). Characterization of a nosocomial outbreak caused by a multi-resistant *Acinetobacter baumannii* strain with a carbapenem-hydrolyzing enzyme: high-level carbapenem resistance in *A. baumannii* is not due solely to the presence of β-lactamases. *J Clin Microbiol*, 38, 3299-305.

16. BOURN J (2000). The Management and Control of Hospital Acquired Infection in Acute NHS Trusts in England is available, *National Audit Office (NAO) press office*, http://www.nao.org.uk/publications/nao_reports/9900230.pdf.

17. BOUVET, P. J. & GRIMONT, P.A. (1986). Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii sp*. nov., *Acinetobacter hae- molyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov., and emended description of *Acinetobacter calcoaceticus* and *Acin- etobacter lwoffii*. *Int. J. Syst. Bacteriol*, 36:228–240.

18. BOUVET, P. J. & GRIMONT, P. A. (1987). Identification and biotyping of clinical isolates of *Acinetobacter*. *Ann Inst Pasteur Microbiol,* 138, 569-78.

19. BOUVET, P. J., JEANJEAN, S., VIEU, J. F. & DIJKSHOORN, L. (1990). Species, biotype, and bacteriophage type determinations compared with cell envelope protein profiles for typing *Acinetobacter* strains. *J Clin Microbiol,* 28, 170-6.

20. BRISOU, J., & PREVOT, A.R. (1954). Studies on bacterialtaxonomy. X. The revision of species under Achromobacter group. *Ann. Inst. Pasteur* (Paris), 86:722–728.

21. BROWN, S. & AMYES, S.G. (2006). OXA β-lactamases in *Acinetobacter*: the story so far. *J Antimicrob Chemother,* 57, 1-3.

22. BROWN, S., YOUNG, H. K. & AMYES, S. G. (2005). Characterisation of OXA-51, a novel class-D carbapenemase found in genetically unrelated clinical strains of *Acinetobacter baumannii* from Argentina. *Clin Microbiol Infect,* 11, 15-23.

23. BSAC (2012). Methods for Antimicrobial Susceptibility Testing. Version 11.1 May http://www.bsac.org.uk/Resources/BSAC/Version%20%2011.1%202012%20Final%2021.5.pdf

24. CARR, E. L., KAMPFER, P., PATEL, B.K., GURTLER, V. & SEVIOUR, R.J. (2003). Seven novel species of *Acinetobacter* isolated from activated sludge. *Int. J.Syst. Evol. Microbiol,* 53:953–963.

25. CDC. (2004). *Acinetobacter baumannii* infections among patients at military medical facilities treating injured U.S. service members, 2002–2004. *MMWR Morb. Mortal. Wkly. Rep,* 53:1063–1066.

26. CHEN, T. L., SIU, L. K., WU, R. C., SHAIO, M. F., HUANG, L. Y., FUNG, C. P., LEE, C. M. & CHO, W. L. (2007). Comparison of one-tube multiplex PCR, automated ribotyping and intergenic spacer (ITS) sequencing for rapid identification of *Acinetobacter baumannii*. *Clin Microbiol Infect,* 13**,** 801-6.

27. CHOI, S. H., CHOO, E. J., KWAK, Y. G., KIM, M. Y., JUN, J. B., KIM, M. N., KIM, N. J., JEONG, J. Y., KIM, Y. S. & WOO, J. H. (2006). Clinical characteristics and outcomes of bacteremia caused by *Acinetobacter* species other than *A. baumannii*: comparison with *A. baumannii* bacteremia. *J Infect Chemother,* 12**,** 380-6.

28. CHU, Y. W., LEUNG, C.M., HOUANG, E.T., Ng, K.C., LEUNG, C.B., LEUNG, H.Y. & CHENG, A.F. (1999). Skin carriage of Acinetobacters in Hong Kong. *J. Clin. Microbiol,* 37:2962–2967.

29. COATES, A., HU, Y., BAX, R. & PAGE, C. (2002). The future challenges facing the development of new antimicrobial drugs. *Natu Rev Drug Disc,* 1, 895-910.

30. COELHO, J., WOODFORD, N., TURTON, J. & LIVERMORE, D. M. (2004). Multiresistant *Acinetobacter* in the UK: how big a threat? *Jour Hosp Infe,* 58, 167-169.

31. COELHO, J. M., TURTON, J. F., KAUFMANN, M. E., GLOVER, J., WOODFORD, N., WARNER, M., PALEPOU, M. F., PIKE, R., PITT, T. L., PATEL, B. C. & LIVERMORE,

D. M. (2006). Occurrence of carbapenernresistant *Acinetobacter baumannii* clones at multiple hospitals in London and southeast England. *J Clin Microbiol,* 44, 3623-3627.

32. DALLA-COSTA, L. M., COELHO, J. M., SOUZA, H. A., CASTRO, M. E., STIER, C. J., BRAGAGNOLO, K. L., REA-NETO, A., PENTEADO-FILHO, S. R., LIVERMORE, D. M. & WOODFORD, N. (2003). Outbreak of carbapenem-resistant *Acinetobacter baumannii* producing the OXA-23 enzyme in Curitiba, Brazil. *J Clin Microbiol,* 41, 3403-6.

33. DOLZANI, L., TONIN, E., LAGATOLLA, C., PRANDIN, L. & MONTIBRAGADIN, C. (1995). Identification of *Acinetobacter* isolates in the *A. calcoaceticus-A. baumannii* complex by restriction analysis of the 16S-23S rRNA intergenic-spacer sequences. *J Clin Microbiol,* 33, 1108-13.

34. DONALD, H.M., SCAIFE, W., AMYES S.G. & YOUNG, H.K. (2000). Sequence analysis of ARI-1, a novel OXA beta-lactamase, responsible for imipenem resistance in *Acinetobacter baumannii* 6B92. *Antimicrobial Agents Chemotherapy*, 44(1):196-9

35. DIJKSHOORN, L., NEMEC, A. & SEIFERT, H. (2007). An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nat Rev Microbiol,* 5, 939-951.

36. EVANS, B.A., BROWN, S., HAMOUDA, A., FINDLAY, J. & AMYES, S.G. (2007). Eleven novel OXA-51-like enzymes from clinical isolates of *Acinetobacter baumannii. Clin Microbiol Infect,* 11, 1137-8.

37. FALAGAS, M. E., BLIZIOTIS, I. A. & SIEMPOS, I.I. (2006). Attributable mortality of *Acinetobacter baumannii* infections in critically ill patients: a systematic review of matched cohort and case-control studies. *Crit Care,* 10**,** 48.

38. FALAGAS, M. E., KARVELI, E. A., KELESIDIS, I. & KELESIDIS, T. (2007). Community-acquired *Acinetobacter* infections. *Eur J Clin Microbiol Infect Dis,* 26**,** 857-868.

39. FOURNIER, P. E., & RICHET, H. (2006). The epidemiology and control of *Acinetobacter baumannii* in health care facilities. *Clin. Infect. Dis,* 42:692–699.

40. GALES, A. C., JONES, R. N., FORWARD, K. R., LINARES, J., SADER, H. S. & VERHOEF, J. (2001). Emerging importance of multidrug-resistant *Acinetobacter* spp and *Stenotrophomonas maltophilia* as pathogens in seriously ill patients: geographic patterns, epidemiological features and trends in the SENTRY antimicrobial surveillance program (1997-1999). *Clin Infect Dis,* 32, 104-113.

41. GALES, A. C., TOGNIM, M.C., REIS, A.O., JONES, R.N. & SADER, H.S. (2003). Emergence of an IMP-like metallo-enzyme in an *Acinetobacter baumannii* clinical strain from a Brazilian teaching hospital. *Diagn. Microbiol. Infect. Dis,* 45:77–79.

42. GENEBANK (2011). OXA-type β-Lactamases,. *Lahey clinic*, http://www.lahey.org/Studies/other.asp#table1.

43. GERNER-SMIDT, P., TJERNBERG, I & URSING, J. (1991). Reliability of phenotypic tests for identification of *Acinetobacter* species. *J. Clin. Microbiol,* 29:277–282.

44. GOUBY,A, CARLES-NURIT, M.J., BOUZIGES, N., BOURG, G., MESNARD, R. & BOUVET. P.J. (1992). Use of pulsed-field gel electrophoresis for investigation of hospital outbreaks of *Acinetobacter baumannii*. *J ClinMicrobiol,* 30, 1588-91.

45. HAWLEY, J. S., MURRAY, C.K., GRIFFITH, M.E., MCELMEEL, M.L., FULCHER, L.C., HOSPENTHAL, D.R. & JORGENSEN, J.H. (2007). Susceptibility of *Acinetobacter* strains isolated from deployed U.S. military personnel. *Antimicrob. Agents Chemother,* 51:376–378.

46. HERITIER, C., POIREL, L., FOURNIER, P. E., CLAVERIE, J. M., RAOULT, D. & NORDMANN, P. (2005). Characterization of the naturally occurring oxacillinase of *Acinetobacter baumannii*. *Antimicrob Agents Chemother,* 49, 4174-4179.

47. HERITIER, C., POIREL, L. & NORDMANN, P. (2006). Cephalosporinase overexpression resulting from insertion of IS*Aba1* in *Acinetobacter baumannii*. *Clin Microbiol Infect,* 12, 123-30.

48. HIGGINS, P. G., WISPLINGHOFF, H., KRUT, O. & SEIFERT, H. (2007). A PCR based method to differentiate between *Acinetobacter baumannii* and *Acinetobacter* genomic species 13TU. *Clin. Microbiol. Infect,* 13:1199–1201.

49. JAMAL, W., SALAMA, M., DEHRAB. N., AlHASHEM, G., SHAHIN, M. & ROTIMI, V.O. (2009). Role of tigecycline in the control of a carbapenem-resistant Acinetobacter baumannii outbreak in an intensive care unit. *J Hosp Infect,* 72(3):234-42.

50. KROEGER, L. A., HOVDE, L. B., MITROPOULOS, I. F., SCHAFER, J. & ROTSCHAFER, J. C. (2007). Colistin methanesulfonate against 191 multidrug-resistant

Acinetobacter baumannii in an in vitro pharmacodynamic model. *Antimicrob Agents Chemother,* 51, 3431-3.

51. KUMAGAI, T., TAMAI, S., ABE, T. & HIKIDA, M. (2002). Current status of oral carbapenem development. *Current Medicinal Chemistry Anti Infective Agents,* 1, 1-14.

52. LAHTI, C. J. (1996). Pulsed field gel electrophoresis in the clinical microbiology laboratory. *J Clin Lab Anal,* 10, 326-30.

53. LIM, Y. M., SHIN, K. S. & KIM, J. (2007). Distinct antimicrobial resistance patterns and antimicrobial resistance-harboring genes according to genomic species of *Acinetobacter* isolates. *J Clin Microbiol,* 45**,** 902-5.

54. LIVERMORE, D. M. (1995). β-lactamases in laboratory and clinical resistance. *Clin Microbiol Rev,* 8, 557-84.

55. LIVERMORE, D. M. (2009). Doripenem: antimicrobial profile and clinical potential. *Diagn Microbiol Infect Dis,* 63**,** 455-458.

56. LIVERMORE, D. M. & WOODFORD, N. (2000). Carbapenemases: a problem in waiting? *Curr Opin Microbiol,* 3, 489-95.

57. LIVERMORE, D. M. & WOODFORD, N. (2006). The β-lactamase threat in *Enterobacteriaceae, Pseudomonas* and *Acinetobacter*. *Trends in Microbiology,* 14, 413-420.

58. LIVERMORE, D. M., MUSHTAQ, S. & WARNER, M. (2009). Activity of the anti-MRSA carbapenem razupenem (PTZ601) against Enterobacteriaceae with defined resistance mechanisms. *J Antimicrob Chemother,* 64: 330-335.

59. MCDONALD, L. C., BANERJEE, S.N. & JARVIS, W.R. (1999). Seasonal variation of *Acinetobacter* infections: 1987–1996. *Clin. Infect. Dis*, 29:1133–1137.

60. MUGNIER, P., POIREL, L. & PITOUT, M. (2008) Carbapenem-resistant and OXA-23-producing *Acinetobacter baumannii* isolates in the United Arab Emirates. *Clinical Microbiology and Infection,* 14:879-82.

61. MUGNIER, P.D., BINDAYNA, K.M., POIREL, L. & NORDMANN, P. (2009) Diversity of plasmid-mediated carbapenem-hydrolysing oxacillinases among carbapenem-resistant *Acinetobacter baumannii* isolates from Kingdom of Bahrain. *Journal of Antimicrobial Chemotherapy*,63:1071-3.

62. NEMEC, A., DE BAERE, T., TJERNBERG, I., VANEECHOUTTE, M., VAN DER REIJDEN, T.J. & DIJKSHOORN, L. (2001). *Acinetobacter ursingii* sp. nov. and *Acinetobacter schindleri* sp. nov., isolated from human clinical specimens. *Int. J. Syst. Evol. Microbiol,* 51:1891–1899.

63. NEMEC, A., DIJKSHOORN, L., CLEENWERCK, I., DE BAERE, T., JANSSENS, D., VAN DER REIJDEN, T. J., JEZEK, P. & VANEECHOUTTE, M. (2003). *Acinetobacter par- vus* sp. nov., a small-colony-forming species isolated from human clinical specimens.

Int. J. Syst. Evol. Microbiol, 53:1563–1567.

64. OBUEKWE, C. O., AL-JADI, Z. K. & AL-SALEH, E. S. (2009). Hydrocarbon degradation in relation to cell-surface hydrophobicity among bacterial hydrocarbon degraders from petroleum-contaminated Kuwait desert environment. *Int Biodeter Biodegr,* 63**,** 273-279.

65. OPAZO, A., SONNEVEND, A., LOPES, B., HAMOUDA, A., GHAZAWI, A., PAL, T. & AMYES, S.G. (2012). Plasmid-encoded PER-7 β-lactamase responsible for ceftazidime resistance in *Acinetobacter baumannii* isolated in the United Arab Emirates. *Journal of Antimicrobial Chemotherapy*, 67(7):1619-22.

66. ORDONEZ, O. F., FLORES, M. R., DIB, J. R., PAZ, A. & FARIAS, M. E. (2009). Extremophile culture collection from Andean lakes: Extreme pristine environments that host a wide diversity of microorganisms with tolerance to UV radiation. *Microbial Ecology,* 58: 461-73.

67. PANTOPOULOU, A., GIAMARELLOS-BOURBOULIS, E. J., RAFTOGANNIS, M., TSAGANOS, T., DONTAS, I., KOUTOUKAS, P., BAZIAKA, F., GIAMARELLOU, H. & PERREA, D. (2007). Colistin offers prolonged survival in experimental infection by multidrug-resistant *Acinetobacter baumannii*: the significance of co-administration of rifampicin. *Int J Antimicrob Agents,* 29, 51-5.

68. PATON, R., MILES, R. S., HOOD, J. & AMYES, S. G. B. (1993). ARI-1: β-lactamases - mediated imipenem resistance in *Acinetobacter baumannii*. *Int. J. Antimicrob. Agents,* 2, 81-88.

69. PEIX, A., LANG, E., VERBARG, S., SPROER, C., RIVAS, R., SANTA-REGINA, I., MATEOS, P. F., MARTINEZ-MOLINA, E., RODRIGUEZ-BARRUECO, C. & VELAZQUEZ, E. (2009). *Acinetobacter* strains IH9 and OCI1, two rhizospheric phosphate solubilizing isolates able to promote plant growth, constitute a new genomovar of *Acinetobacter calcoaceticus*. *Syst Appl Microbiol,* 32**,** 334-341.

70. PELEG, A. Y., FRANKLIN, C., WALTERS, L. J., BELL, J. M. & SPELMAN, D.W. (2006). OXA-58 and IMP-4 carbapenem-hydrolyzing β-lactamases in an *Acinetobacter junii* blood culture isolate from Australia. *Antimicrob Agents Chemother,* 50**,** 399-400.

71. PELEG, A.Y., SEIFERT, H. & PATERSON, D.L. (2008). Acinetobacter baumannii: emergence of a successful pathogen. *Clinical microbiology review,* 21(3):538-82.

72. PEREZ, F., HUJER, A.M., HUJER, K.M., DECKER, B.K., RATHER, P.N. & BONOMO, R.A. (2007). Global challenge of multidrug-resistant *Acinetobacter baumannii. Antimicrobial Agents and Chemotherapy,* 51:3471-84.

73. PESTKA, S. (1971). Inhibitors of ribosome functions. *Annu Revi in Microbiol,* 25,487-562.

74. PFEIFER, Y., WILHARM, G., ZANDER, E., WICHELHAUS, T.A., GÖTTIG, S., HUNFELD, K.P., SEIFERT, H., WITTE, W. & HIGGINS, P.G. (2011). Molecular characterization of blaNDM-1 in an *Acinetobacter baumannii* strain isolated in Germany in 2007. *Journal of Antimicrobical Chemotherapy,* 66: 1998–2001

75. PIERONI, P. (1997). Antimicrobial susceptibilities of blood culture isolates obtained before and after the introduction of ciprofloxacin. *Br Soc Antimicrob Chemo,* 39, 419-422.

76. POIREL, L., MARQUE, S., HERITIER, C., SEGONDS, C., CHABANON, G. & NORDMANN, P. (2005). OXA-58, a novel class D β-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. *Antimicrob Agents Chemother,* 49, 202-208.

77. POIREL, L. & NORDMANN, P. (2006a). Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clinical Microbiology and Infection,* 12:826-36.

78. POIREL, L. & NORDMANN, P. (2006b). Genetic structures at the origin of the acquisition and expression of the carbapenem-hydrolyzing oxacillinase gene *bla*OXA-58 in *Acinetobacter baumannii*. *Antimicrob Agents Chemother,* 50,1442-8.

79. PONTIROLI, A., RIZZI, A., SIMONET, P., DAFFONCHIO, D., VOGEL, T. M. & MONIER, J. M. (2009). Visual evidence of horizontal gene transfer between plants and bacteria in the phytosphere of transplastomic tobacco. *Appl Environ Microbiol,* 75, 3314-3322.

80. QUEENAN, A. M. & BUSH, K. (2007). Carbapenemases: the versatile beta-lactamases. *Clin. Microbiol. Rev,* 20:440–458.

81. ROSSAU, R., VAN LANDSCHOOT, A., GILLIS, M. & DE LEY, J. (1991). Taxonomy of *Moraxellaceae* fam. nov., a new bacterial family to accomodate the genera *Moraxella*, *Acinetobacter*, and *Psychrobacter* and related organisms. *Int. J. Syst. Bacteriol,* 41:310–319.

82. SCOTT, P,. DEYE, G,. SRINIVASAN, A,. MURRAY, C,. MORAN, K,. HULTEN, E,. FISHBAIN, J,. CRAFT, D,. RIDDELL, S,. LINDLET, L,. MANCUSO, J,. MILSTREY, E,. BAUTISTA, C.T., PATEL, J,. EWELL, A,. HAMILTON, T,. GADDY, C,. TENNEY M,. CHRISTOPHER, G,. PETERSEN, K,. ENDY, T. & PETRUCCELLI, B. (2007). An outbreak of multidrug-resistant *Acinetobacter baumannii-calcoaceticus* complex infection in the US military health care system associated with military operations in Iraq. Clinical Infectious Diseases, 15;44(12):1577-84.

83. SEIFERT, H., DIJKSHOORN, L., GERNER-SMIDT, P., PELZER, N., TJERNBERG, I. & VANEECHOUTTE, M. (1997). Distribution of *Acinetobacter* species on human skin: comparison of phenotypic and genotypic identification methods. *J. Clin. Microbiol,* 35:2819–2825.

84. SEIFERT, H., SCHULZE, A., BAGINSKI, R. & PULVERER, G. (1994a). Comparison of four different methods for epidemiologic typing of *Acinetobacter baumannii*. *J Clin Microbiol,* 32, 1816-9.

85. SEIFERT, H., STRATE, A., SCHULZE, A. & PULVERER, G. (1994b). Bacteremia due to *Acinetobacter* species other than *Acinetobacter baumannii*. *Infection,* 22, 379-385.

86. SEIFERT, H., DOLZANI, L., BRESSAN, R.,VAN DER REIJDEN, T.,VAN STRIJEN, B., STEFANIK, D., HEERSMA, H. & DIJKSHOORN, L. (2005). Standardization and interlaboratory reproducibility assessment of pulsed-field gel electrophoresisgenerated fingerprints of *Acinetobacter baumannii*. J. Clin. Microbiol, 43:4328–4335.

87. SMITH, J. T. (1986). The mode of action of 4-quinolones and possible mechanisms of resistance. *J Antimicrob Chemother,* 18, 21-9.

88. SPRATT, B. G. & CROMIE, K. D. (1988). Penicillin-binding proteins of gramnegative bacteria. *Rev Infect Dis,* 10, 699-711.

89. TENOVER, F. C., ARBEIT, R.D., GOERING, R.V., MICKELSEN, P.A., MURRAY, B.E., PERSING, D.H. & SWAMINATHAN, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol,* 33: 2233–2239.

90. TOWNER, K. J., BERGOGNE-BÉRÉZIN, E. & FEWSON, C. A. (1991). The Biology of *Acinetobacter*: Taxonomy, Clinical Importance, Molecular Biology, Physiology, *Industrial Relevance,* Paris, Springer.

91. TURTON, J. F., KAUFMANN, M. E., GLOVER, J., COELHO, J. M., WARNER, M., PIKE, R. & PITT, T. L. (2005). Detection and typing of integrons in epidemic strains of *Acinetobacter baumannii* found in the United Kingdom. *J Clin Microbiol,*43, 3074-82.

92. TURTON, J. F., WOODFORD, N., GLOVER, J., YARDE, S., KAUFMANN, M.E. & PITT, T.L. (2006). Identification of *Acinetobacter baumannii* by detection of the *bla*OXA-51-like carbapenemase gene intrinsic to this species. *J. Clin. Microbiol,* 44:2974–2976.

93. TURTON, J. F., GABRIEL, S. N., VALDERREY, C., KAUFMANN, M. E. & PITT, T. L. (2007). Use of sequence-based typing and multiplex PCR to identify clonal lineages of outbreak strains of *Acinetobacter baumannii*. *Clin Microbiol Infect,* 13, 807-15.

94. URBAN, C., GO, E., MARIANO, N., BERGER, B. J., AVRAHAM, I., RUBIN, D. & RAHAL, J. J. (1993). Effect of sulbactam on infections caused by imipenem-resistant *Acinetobacter calcoaceticus* biotype anitratus. *J Infect Dis,* 167, 448-51.

95. VALENCIA, R., ARROYO, L. A., CONDE, M., ALDANA, J. M., TORRES, M. J., FERNANDEZ-CUENCA, F., GARNACHO-MONTERO, J., CISNEROS, J. M., ORTIZ, C., PACHON, J. & AZNAR, J. (2009). Nosocomial outbreak of infection with pan-drug-resistant *Acinetobacter baumannii* in a tertiary care University hospital. *Infection Control and Hospital Epidemiology,* 30**,** 257- 263.

96. VAN DESSEL, H., DIJKSHOORN, L., VAN DER REIJDEN, T., BAKKER, N., PAAUW, A., VAN DEN BROEK, P., VERHOEF, J. & BRISSE, S. (2004). Identification of a new geographically widespread multiresistant *Acinetobacter baumannii* clone from European hospitals. *Res Microbiol,* 155**,** 105-112.

97. WALTHER-RASMUSSEN, J. & HOIBY, N. (2006). OXA-type carbapenemases. *J Antimicrob Chemother,* 57, 373-83.

98. WEHRLI, W., KNUSEL, F., SCHMID, K. & STAEHELIN, M. (1968). Interaction of rifamycin with bacterial RNA polymerase. *Proceed of the Nati Acad of Scie,* 61, 667-673.

99. WISPLINGHOFF, H., BISCHOFF, T., TALLENT, S.M., SEIFERT, H., WENZEL, R.P. & EDMOND, M.B. (2004). Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis,* 39:309–317.

100. YU, Y. S., YANG, Q., XU, X. W., KONG, H. S., XU, G. Y. & ZHONG, B. Y. (2004). Typing and characterization of carbapenem-resistant *Acinetobacter calcoaceticus-A. baumannii* complex in a Chinese hospital. *J Med Microbiol,* 53, 653.

101. YUN, H. C., MURRAY, C.K., ROOP, S.A., HOSPENTHAL, D.R., GOURDINE, E. & DOOLEY, D.P. (2006). Bacteria recovered from patients admitted to a deployed U.S. military hospital in Baghdad, Iraq. *Mil. Med,* 171:821–825.